

MICROBIAL COMMUNITY DYNAMICS OF THE DEEPWATER HORIZON OIL SPILL

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ABSTRACT

Tingting Yang: Microbial Community Dynamics of the Deepwater Horizon Oil spill
(Under the direction of Andreas P. Teske)

The *Deepwater horizon* (DWH) oil spill released ~4.9 million (780,000 m³) barrels of crude oil into the Gulf of Mexico, causing the worst environmental disaster in U.S. history. Over 50% of the released oil could not be recovered. The task of biodegradation, to recycle the huge amount of hydrocarbon back to inorganic carbon and into microbial biomass, fell to the bacterial communities of the water column and the seafloor– which consequently changed in response to the oil fallout. These compositional and functional changes of the bacterial community in different stages of the spill provide the main focus of my study.

My PhD project includes time series observation of the oil contaminated water column as well as the sediment of the Gulf of Mexico. The crude oil from the riser pipe at the seafloor (~1500 m depth) formed a deep sea hydrocarbon plume, as well as huge amount of surface oil slick. An uncultured *Oceanospirillales* group and the polycyclic aromatic hydrocarbon (PAH) degrader *Cycloclasticus* were extremely dominant during the spill within the plume and the oil slick during the spill, respectively. After the wellhead was capped, the plume could not be detected; however, *Cycloclasticus* was continuously found in post-spill water. The surface oil slicks formed mucus rich oily marine snow aggregates via the activities of EPS producers such as *Halomonas*. Inside the aggregates several hydrocarbon degraders (i.e. *Cycloclasticus*) and heterotrophic bacteria such as *Roseobacter* bloomed. These oil snow aggregates sank and

eventually made their way to the seafloor, as sediments with oily surface layer has been recovered since September 2010. Bacterial dynamics within the oil contaminated sediment included the appearance of *Roseobacter* and Verrucomicrobiaceae in September 2010, increase of anaerobic sulfate-reducing bacteria and organic matter degrading *Cytophaga* in October 2010. The *Planctomycetes* increased from low clone library proportions in October 2010 towards higher representation in November 2010 and July 2011, one year after the oil spill. Notably, *Cycloclasticus* was detected in the oil contaminated sediment from September to November 2010, strongly supporting the hypothesis of precipitation of oily marine snow aggregates. Besides these molecular observations, novel species of oil-degrading bacteria and potential hydrocarbon and/or organic matter degraders were isolated or enriched from the water samples (plume and surface oil slick) as well as the seafloor sediments. This time series study reveals development of the oil-degrading community together with continuous movement of the release oil from the deep ocean and sea surface to the sediment, via precipitation of the oily snow particles, demonstrates the coincidence of the oil decomposition with its continuing microbial processing.

This dissertation is dedicated to my parents.

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LIST OF ABBREVIATIONS

DWH – Deepwater Horizon

EPS – Exopolysaccharide

GOM – Gulf of Mexico

PAH – Polycyclic aromatic hydrocarbon

PCR – Polymerase chain reaction

SRB – Sulfate-reducing bacteria

INTRODUCTION

The Gulf of Mexico is a major oil reservoir in the United States, with its offshore oil production accounts for 17% of total US crude oil production ([www. eia.gov](http://www.eia.gov)). In total, the Gulf of Mexico has at least 22,000 detected natural seeps (<http://1.usa.gov/1aKybyq>), with estimated 1500 and 3800 barrels of oil naturally released to the Gulf daily, or up to 604,150 liters every year (MacDonald, 1998). Advanced technology allowed deep sea drilling which bring bloom of economy at the risk of the ocean and coastal ecosystems. The nearest example is the Deepwater Horizon oil spill, the worst environmental disaster in US history. On April 20, 2010, high-pressure gas escaped and was released on the drilling rig where it ignited and exploded. 11 workers tragically lost their lives; the Deepwater Horizon drilling rig, located in the northern Gulf of Mexico (28 44. 12 N, 88 23.14 W) about 50 miles south east of the Mississippi Delta (Figure 1), burned and ultimately sank 2 days after the explosion.

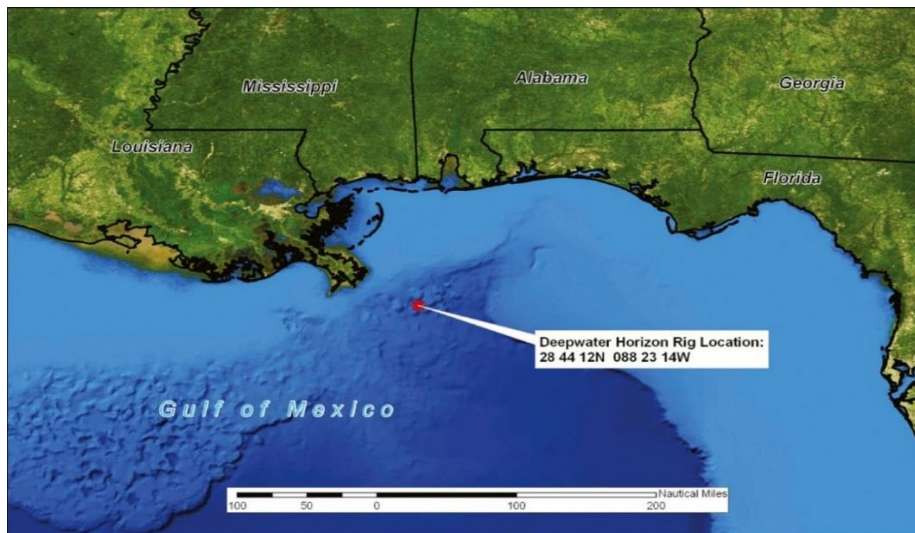


Figure 1. Location of the Deepwater Horizon spill wellhead. ~50 miles southeast of the Mississippi Delta. From (McNutt et al. 2011).

Through the broken riser pipe, 50000-70000 barrels of oil were released per day. It took 87 days to stop the flow of oil from the wellhead; The estimated minimum rate of discharge was 58000 barrels oil per day (MacDonald, 2010); total oil released into the Gulf of Mexico was about 4.9 million barrels as calculated by the National Incident Command's Flow Rate Technical Group (McNutt *et al.*, 2011). Together with a large volume of crude oil, the spill released a huge amount of natural gas as well (Valentine *et al.*, 2010, Joye *et al.*, 2011, Reddy *et al.*, 2012). Analysis of original discharge samples directly from the damaged riser pipe at ~ 1500 meter below the sea surface showed that the oil fraction contained 74% saturated hydrocarbons, 16% aromatic hydrocarbons, and 10% polar hydrocarbons; the C1-C5 hydrocarbon gases consisted of 82.5% ($\sim 1.0 \times 10^{11}$ g) methane, 8.3% ($\sim 1.9 \times 10^{10}$ g) ethane, and 5.3% ($\sim 1.8 \times 10^{10}$ g) propane (Reddy *et al.*, 2012). The wellhead *in situ* low temperature (about 4 °C), high pressure (~15 atm) together with the complex oil components with various physical and chemical properties would led to stratified oily layers in the water column (Figure 2). Primarily soluble mixture such as light alkanes were trapped in the deep ocean and formed hydrocarbon plume; insoluble, non-volatile mixture (PAHs) were able migrate up to the sea surface, formed enormous oil slick; insoluble but volatile mixture such as some alkanes and aromatic compounds evaporated to the atmosphere (Ryerson *et al.*, 2012). The surface slicks formed oily marine snow aggregates after a short period (Passow *et al.*, 2012); part of these oil aggregates sank down to the seafloor after losing their buoyancy, dramatically changed the benthic environment dwells worms, deep sea coral, fish and other benthonic organisms. A large portion of surface oil was washed onshore of Louisiana, Mississippi, Alabama and Florida; the maximum extent of shoreline oiling involved almost 1,100 miles of shoreline (Ramseur & Hagerty, 2014). These environments where dwelled most of the released oil are tightly connected, however, their distinct physical, chemical,

geological and biological characteristics lead to different weathering processes and diversified residues. The complexity of oil components together with the Gulf currents matrix made it extremely hard to continuously track the transportation of the released oil and monitor its component change.

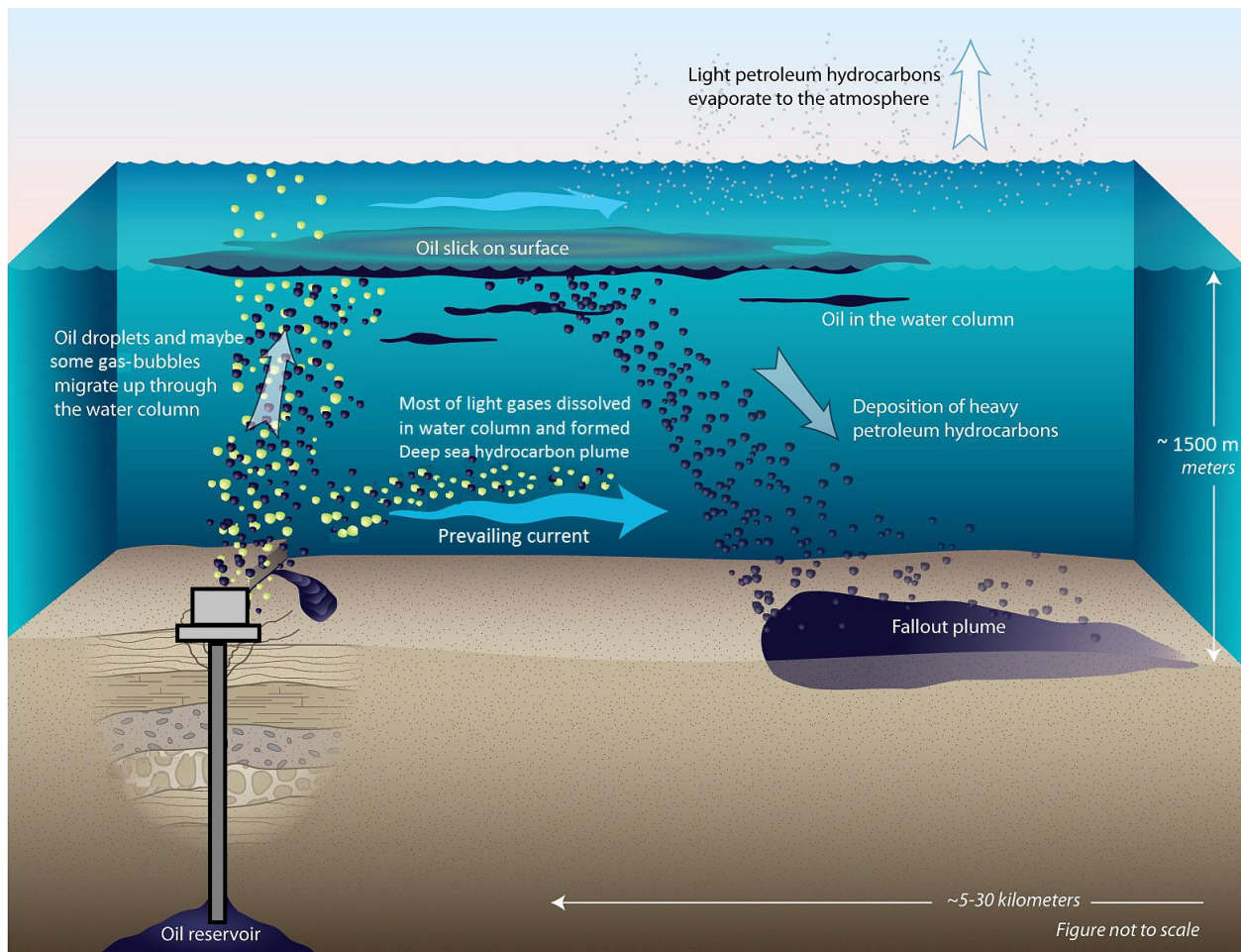


Figure 2. Graphic depiction of Deepwater Horizon spill. Modified from Jack Cook, WHOI.

Oil and gas in the deep ocean water column

The DWH oil and gas leaked from a well 1500 m below the surface of the ocean, where the temperature is about 5 °C. The interplay of gas and oil in multiphase flow, preferential solubility of each oil constituent and other factors together formed a deep “plume” at depth of

1000 m to 1300 m (Camilli *et al.*, 2010, Diercks *et al.*, 2010, Hazen *et al.*, 2010, Joye *et al.*, 2011). The first time that the deepwater plume was observed was during the May 2010 R/V Pelican cruise (May 9-16, 2010) (Diercks *et al.*, 2010), where the equipped CDOM (color dissolved organic matter) sensor detected extremely high concentration of dissolved organic matter in the deep ocean, coincident with the dissolved oxygen (DO) anomaly at the same depth (Figure 3). After then, the plume had been reported in many other cruises from May to late June, 2010. Pictures took by underwater camera clearly showed the specific plume layer was dramatically different by its milky color, presumably caused by the tiny crystals formed by light hydrocarbons at the in situ temperature and pressure.

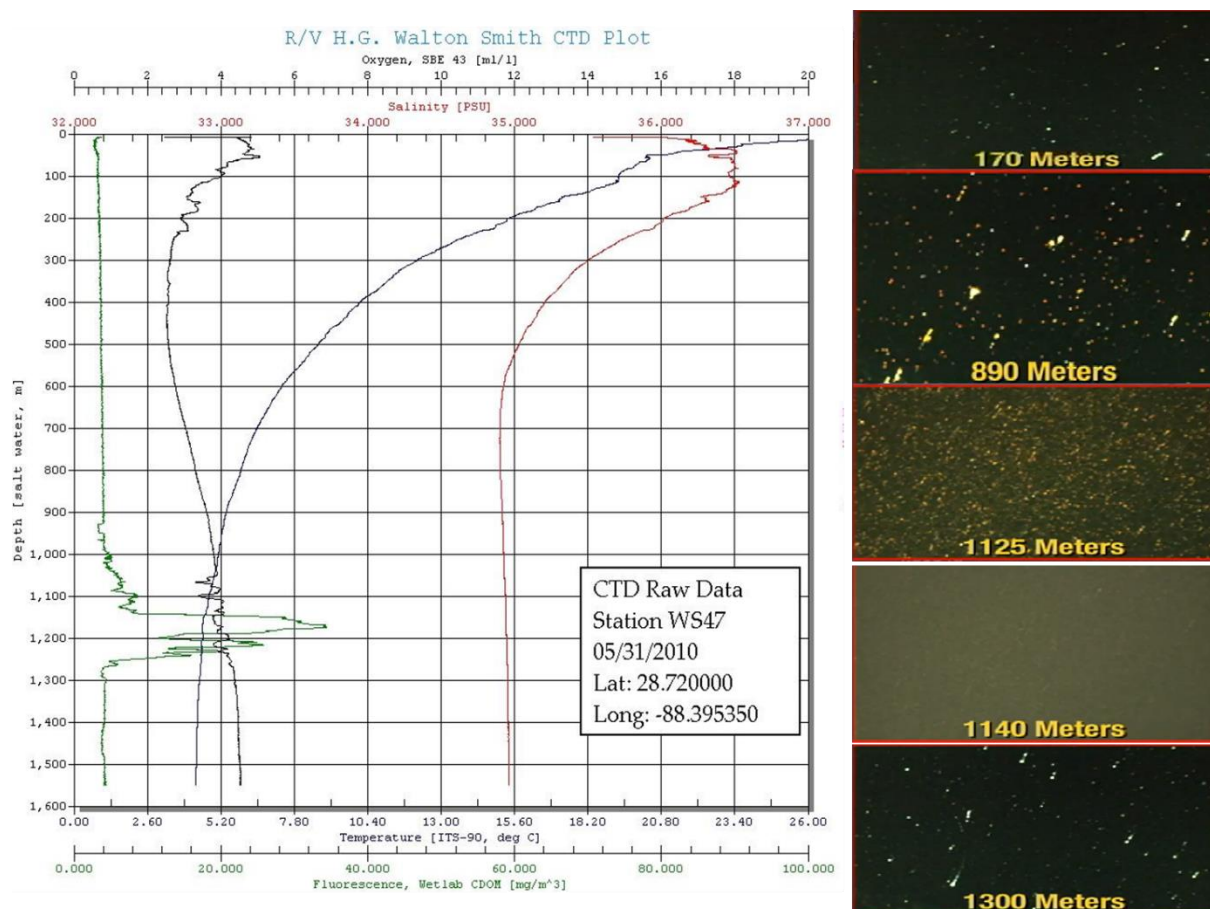


Figure 3. CTD profile shows a plume at depth 1100 – 1270 m with increased CDOM and decreased oxygen concentration. Pictures at right were taken by underwater camera, showing

morphology change of oil in the water column at various depths (Pics from Vernon Asper, RV Walton Smith, May 2010)

The deep plume was comprised of dissolved hydrocarbons and small droplets of oil (less than a micron) (Ryerson *et al.*, 2012). Although readily soluble hydrocarbons made up ~25% of the leaking mixture by mass, it made up ~69% of the deep plume mass (Ryerson *et al.*, 2012). Most of the C1-C3 hydrocarbons and a significant fraction of the water-soluble aromatic compounds were retained in the plume layer, and methane (0.15 gg-1 of reconstituted fluid) was the largest portion of the gaseous phase retained in water (82.5% of the released gas phase) (Reddy *et al.*, 2012). In contrast to other surface oil spills, only 0.01% of the methane injected into the water was released into the atmosphere (Ryerson *et al.*, 2011), which indicated that most of the methane was retained in the water column. In May 2010, the average methane concentration was 1.7 μM southwest of the wellhead (Crespo-Medina *et al.*, 2014). Besides methane, components of the deep hydrocarbon plume also included ethane, propane and light aromatic hydrocarbons, like benzene, toluene, ethylbenzene and total xylenes (referred to collectively as BTEX) (Reddy *et al.*, 2012). Dissolved hydrocarbons were relatively depleted after a while, and then the initially less abundant soluble species accumulated in a relatively high ratio in the plume. The small oil droplets within the plume accounted for 13-43% of the whole plume mass initially transported in the deep ocean (Ryerson *et al.*, 2012).

The plume was moving southwest of the wellhead driven by the currents and the flow was tracked by CDOM concentration and DO anomaly by enormous CTD casts. Even after the wellhead was killed, there were still weak oxygen anomaly was observed hundreds miles away more than five months after the wellhead was killed (Figure 4 from Mission Guidance, NOAA, 2010). Methane concentration decreased sharply from July to September 2010. Average methane

concentration from three cruises from late August to early September was 1.4 ± 2.0 nM, with the maximum concentration (20.4 nM) not exceeding ambient levels for the Gulf of Mexico (Kessler *et al.*, 2011). In contrast, another study showed the August/September methane concentrations remained elevated throughout the water column at sites north of the Macondo wellhead (for

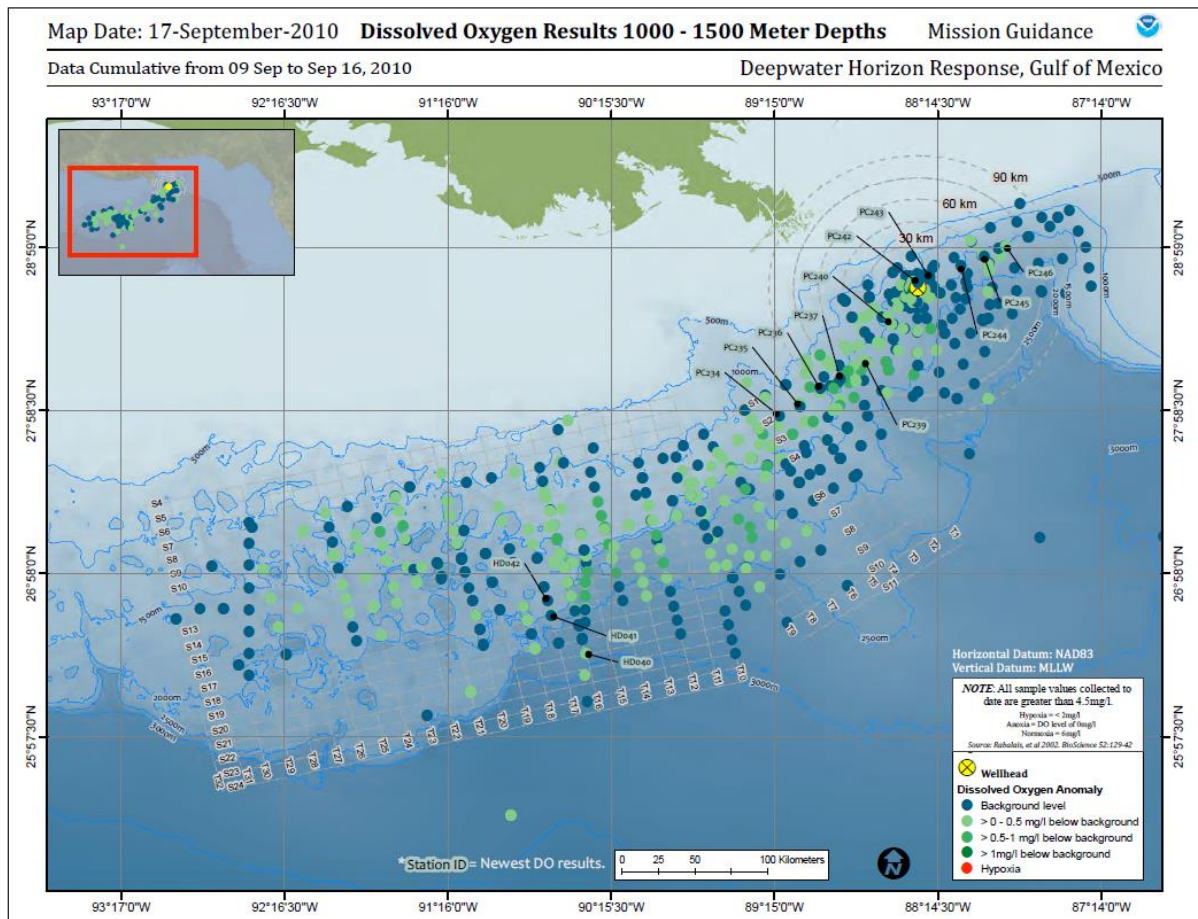


Figure 4. Dissolved oxygen anomalies southwest to the wellhead in September. (Mission Guidance, NOAA 2010, unpublished)

example, a 100nM methane anomaly was observed at MC118 at 650min September 2010) but not to the south/southwest, where low concentrations and low turnover rates of methane at plume depths (900 ~ 1,300 m) were observed, consistent with previously reported observations (Crespo-Medina *et al.*, 2014). Other light hydrocarbon concentration were below detection

(Crespo-Medina *et al.*, 2014). According, the minimum dissolved oxygen concentration observed was 3.7 mg/L in late July; depressions ranged from 0.14 mg/L to 3.7 mg/L below background (Zukunft, 2010). These DO depressions coincided with relative maxima in fluorescence profiles that were interpreted as proxies for hydrocarbons, and extended as far as 80 km from the wellhead. The DO-removing potential in the deep plume was calculated as 0.041 ± 0.008 moles of O₂ per gram of hydrocarbon. About $(3.5 \pm 0.5) \times 10^{10}$ moles O₂ were consumed during the duration of the spill (Ryerson *et al.*, 2012). However, as dissolved oxygen concentrations were not approaching hypoxic levels (<2.0 mg/L) and did not appear to be decreasing over time, their analysis indicated that hypoxic conditions would not occur in association with the deep dispersed plume (Zukunft, 2010).

Beside the released oil, ~771,000 gallons of dispersant (Corexit 9527 and Corexit 9500) was directly applied at the broken riser pipe. It was the first time in history to inject dispersant in the deep ocean. The concentration of the anionic surfactant DOSS (dioctyl sodium sulfosuccinate, major component of Corexit complex) in subsurface samples did not exceed 40 µg L⁻¹ (Gray *et al.*, 2014). By tracing DOSS throughout spill and post-spill time, Kujiawinski *et al.* showed that DOSS was restrained at the plume depth, transported and diluted conservatively as indicated by its concentration distribution, and it persisted more than 300 km from the wellhead, 64 days after deepwater dispersant applications ceased (Kujawinski *et al.*, 2011).

It is believed that biodegradation was the most important means in regulating the fate of hydrocarbons in the deep marine environment. Many studies confirmed that in the early stage (May to early June, 2010) the plume highly enriched a novel *Oceanospirillales* group, which constituted more than half of the bacteria community (Hazen *et al.*, 2010, Redmond & Valentine, 2011, Yang *et al.*, 2014). Metagenomic data indicated the *Oceanospirillales* obtains genes

involved in cycloalkane degradation (Mason *et al.*, 2012), presumably be the major group for degrading light alkanes in the plume. Other oil degraders, such as *Colwellia*, *Marinobacter*, and *Cycloclasticus* were found as well, but at much lower abundance. While the bacteria community structure changed in late June, *Colwellia* and *Cycloclasticus* became the dominant groups (Valentine *et al.*, 2010). Interestingly, the primary drivers of microbial respiration (as measured in the deep plume in June 11-21, 2010) were propane (C_3H_8) and ethane (C_2H_6), instead of methane, which had extremely high concentration compared to other gases according to Valentine's group (Valentine *et al.*, 2010). Oxygen consumed by microbial consumption of propane and ethane accounted for 70% of the observed oxygen anomalies in the plume according to Valentine *et al.* (Valentine *et al.*, 2010). Surprisingly, the peak of Methanotrophs was not detected in Valentine's study even though the plume methane disappeared in a short time after the wellhead was capped. In contrast, another study showed in early May 2010 the measured methane-oxidation rates ranged from 0.014 to 502 $nM\ d^{-1}$ and were highest in the deepwater plumes (Crespo-Medina *et al.*, 2014). Methane-oxidation rates increased to maximal of 5,900 $nM\ d^{-1}$ in late May/early June, but dropped to tens to hundreds of $nM\ d^{-1}$ in late June, although methane concentrations were still above background (tens of μM on average), and continued to drop to 3-5 $nM\ d^{-1}$ when measured in December (Crespo-Medina *et al.*, 2014). Putatively the plume methane was predominately consumed by a novel phylotype of Methanotrophs, together with canonical methanotrophs; since the peak in abundance of the *pmoA* (particulate methane monooxygenase) genes in May and early June corresponded to the maximum methane-oxidation rate (Crespo-Medina *et al.*, 2014). These results were supported by the abundance of transcripts with homology to the novel *pmoA* sequence in the transcriptome of samples collected contemporaneously (Rivers *et al.*, 2013). The specific metabolic capabilities of the new

phylotype of Methanotrophs is not known; the affinity for methane of the novel phylotype was assumed low, since methanotropic activity and *pmoA* gene abundance sharply declined during June/July together with the sudden decrease in methane concentration (Crespo-Medina *et al.*, 2014). In the results of another functional gene survey, *pmoA* and *mmoX* (soluble methane monooxygenase genes) were both found within plume and non-plume samples (Lu *et al.*, 2012).

For the fate of methane in the plume, Kessler *et al.* claimed that methanotrophic and methylotrophic gammaproteobacteria had consumed all dissolved methane from the *Deepwater Horizon* incident, as these two groups of bacteria were found in 16S rRNA clone libraries in their August to September water samples. Yet, the presence of these two bacterial groups can also be interpreted as evidence of bacterial consumption of high molecular weight dissolved organic matter (McCarren *et al.*, 2010). Although naturally enriched inside plume, the conspicuous *Oceanospirillales* escaped from all tempt of pure culturing; novel species of traditional oil and gas consuming bacteria *Colwellia*, *Marinobacter* and *Cycloclasticus* were isolated from plume water under cool temperature (Baelum *et al.*, 2012, Gutierrez *et al.*, 2013, Gutierrez *et al.*, 2013). For all the potential oil degraders, the question of which bacterial group was the most active degrader had remained open. Various metabolic genes involved both in aerobic and anaerobic oil degradation were found to be more abundant within the plume than outside the plume, indicating a whole microbial community response that was not limited to the novel *Oceanospirillales* (Lu *et al.*, 2012).

Biodegradation not only decreased the oxygen concentration, but would also consumed large amount of the main nutrients (e.g. nitrogen and phosphorus) and trace elements such as Fe, Cu, Mn etc. Nitrogen is essential to the growth of microbes; under circumstances of oil spill, the enormous carbon sources drive fast microbial reamplification, makes nitrogen as limiting

resource to the growth of bacteria (Yakimov *et al.*, 2007). Those bacteria can utilize maximum amount of nitrogen using various strategies (storage, low threshold of nitrogen facilitation concentration, fast nitrogen turnover time) would survive and bloom (Yakimov *et al.*, 2007). However, the NH_4 concentration in the Gulf of Mexico water column slightly increased from ~ 1 μM background level to dozens of μM on average during the spill period; no obvious change was observed in NO_x and PO_4 throughout the spill time (Crespo-Medina *et al.*, 2014). Instead, nitrite reductase transcripts suggested that nitrite was a major source of N other than ammonium or nitrate in the plume microbial population (Rivers *et al.*, 2013). Since the main nutrients (nitrogen and phosphorus) were present in sufficiently high concentration in the plume, it is more likely that the trace elements (Fe, Cu etc.), virus lysis and the protist predators contribute to the decrease of the methanotrophs (Joung & Shiller, 2013, Crespo-Medina *et al.*, 2014).

Oil and gas in surface of ocean and air, and the oily marine snow

As was first seen two days after the explosion of the Deepwater Horizon oil rig, the surface of the North Gulf of Mexico was heavily contaminated by insoluble and non-volatile surface slicks and oil sheen (Figure 5). The estimated area of surface slick (plus oil sheen) was about 1759 square miles (MacDonald, 2010). The amount of oil on the sea surface on May 17 was between 129000 and 246000 barrels (Clark, 2010). Subsurface chemical data show that $\sim 31\%$ of the leaking gas and oil was initially transported in the form of oil droplets up to surface of the ocean and the overlying atmosphere (Ryerson *et al.*, 2011, Ryerson *et al.*, 2012). A mean buoyant velocity calculated by Ryerson *et al.* was at least 0.05 m/s from the seafloor (1500 m), implying a mean vertical transport time of no more than 10 hours for insoluble oil droplets (millimeter-scale diameters) surfacing from the seafloor (Ryerson *et al.*, 2012). In the surfacing mixture, about 14% were volatile or semivolatile and were capable of reaching the surface,

where they evaporate from the slick within 1-2 d of surfacing (Ryerson *et al.*, 2011, Ryerson *et al.*, 2012); ~458000 kg/day hydrocarbons were evaporating from the ocean surface (Ryerson *et al.*, 2011). n-C17, n-C16, n-C18, and n-C15 were the four most abundant hydrocarbons by mass in the initial surface slick; the four most abundant hydrocarbons by mass in the evaporating mixture were n-heptane, n-octane, n-nonane and methylcyclohexane (Ryerson *et al.*, 2012). However, methane, ethane, and benzene, which were abundant in the deep plume, were not released into the atmosphere, indicating complete dissolution in the water column (McNutt *et al.*, 2011, Ryerson *et al.*, 2011, Ryerson *et al.*, 2012). Based on data from 10 June, 2010, the hydrocarbons released as surface slick and evaporating into overlying air were estimated as 1.0 ± 0.5 and 0.46 ± 0.1 million kg/day, respectively, as shown in Figure 6. These surface slicks collected up to 240 km from the wellhead showed losses of compounds eluting earlier than n-C₁₃, consistent with evaporative loss (Aeppli *et al.*, 2012). In addition, dissolution affected these early weathering-stage slicks as indicated by the depletion of naphthalene relative to C1-naphthalene as compared to the oil directly from the wellhead; however, longer chain alkane degradation was not observed at that time as reflected by constant n-C₁₈/phytane ratio (Aeppli *et al.*, 2012). The total concentration of 30 PAHs decreased from 18 mg g⁻¹ oil for the wellhead to 7.7-13 mg g⁻¹ oil for surface slicks, and smaller PAHs (e.g. naphthalene and phenanthrene) were depleted presumably due to their higher water solubility (Aeppli *et al.*, 2012).



Figure 5. Surface oil slicks from May 2010 R/V Pelican cruise. (Credit: Luke McKay)

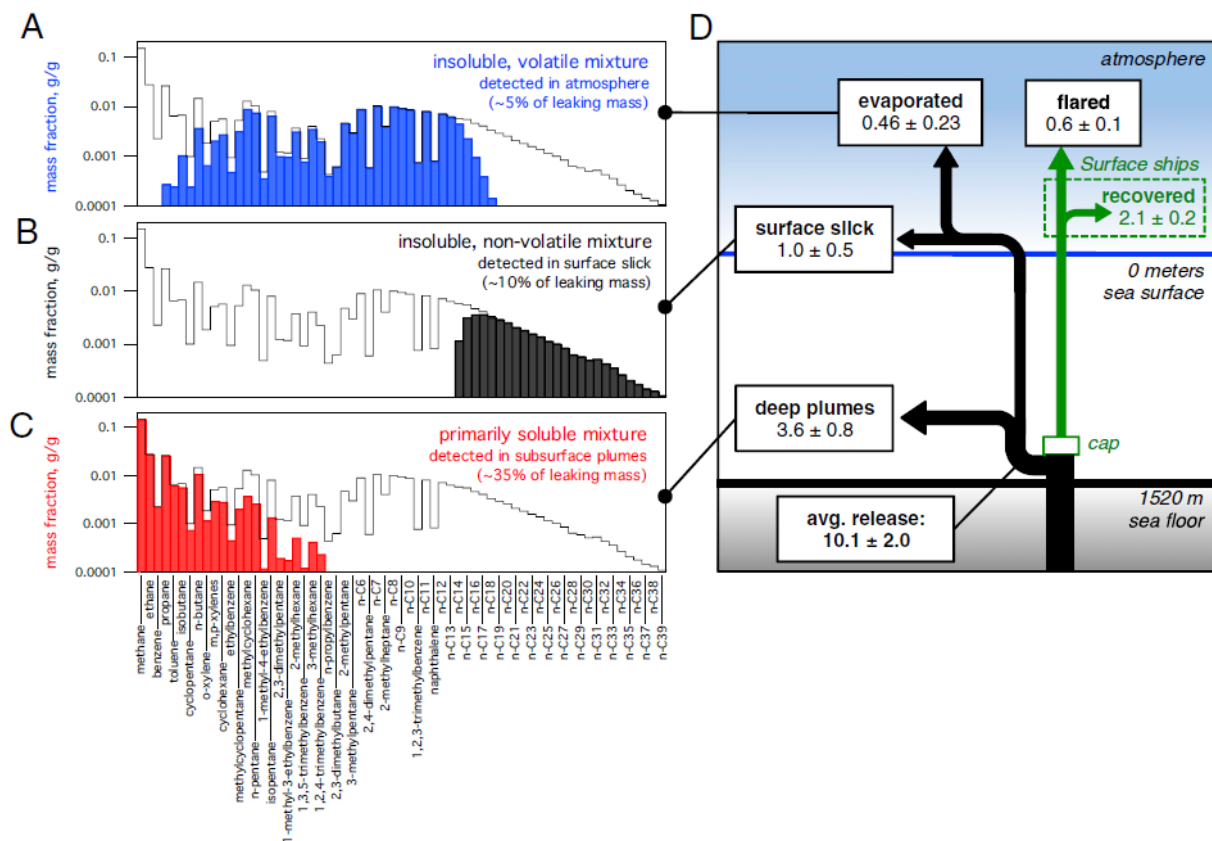


Figure 6. Evaporated hydrocarbon composition after 2 d (A; blue bars), surface oil slick composition after 2 d (B; black bars), and dissolved hydrocarbon composition (C; red bars). The leaking hydrocarbon composition from CH₄ through n-C₃₉ (black line) is shown in each panel for comparison. (D) Schematic (not to scale) of hydrocarbon mass flows in the marine environment; values are calculated for June 10, 2010, in millions of kilograms per day. From (Ryerson et al. 2012)

Enhancing microbial petroleum degradation with dispersants is a major strategy for bioremediation. During the *Deepwater Horizon* oil spill, approximately 1.4 million gallons of dispersant were used at the surface, between May 15 and July 12 (from <http://www.whitehouse.gov/blog/issues/deepwater-bp-oil-spill>). Dispersants are a mixture of surfactants and hydrocarbon-based solvents (Kujawinski *et al.*, 2011). Indeed, the primary function of dispersant is breaking big oil droplets into smaller ones to enlarge their surface areas, i.e. emulsification. By this means, dispersant helps microbes to attach to oil droplets for

enzymatic biodegradation (Pavitrana *et al.*, 2006). However, in a lab microscopy experiment, researchers have found that the alkane degrader *Alcanivorax* tended to attach on the big oil droplets instead of smaller ones (Roman, 2014). Another study indicated that the dispersant increases the toxicity of crude oil to marine zooplankton (Almeda *et al.*, 2014). Other than by dispersed, the surface slick can be weathered by wind, waves and solar irradiation (Council, 2003). Burning was also a way to remove surface slick during the oil spill, with its obvious side effect of air pollution, and contribution to the extra particle organic matter input to the seafloor (Brooks *et al.*, 2014).

Previous study showed that the biodegradation of the *Deepwater Horizon* oil in the offshore oligotrophic surface water was largely enhanced by the oil input, although both chemical data and enzymatic evidence indicated that phosphate was scarce at the sampling region. No concomitant increase of microbial abundance or biomass was observed in the slick (Edwards *et al.*, 2011). Studies of previous oil spill or lab experiments showed that bacteria belong to *Cycloclasticus* and *Alcanivorax* were dominant groups in oil contaminated sea water (Kasai *et al.*, 2002a, Kasai *et al.*, 2002b, Maruyama *et al.*, 2003, Harayama *et al.*, 2004). These groups most likely contributed to biodegradation in the surface oil slick. In the surface oil slick sampled in May 2010, *Cycloclasticus* dominated (> 90%) the slick bacterial community (Yang *et al.*, 2014); in comparison, the oil slick from June 2010 contained mostly *Pseudoalteromonas* in one sample, but *Pseudomonas*, *Vibrio*, *Acinetobacter*, and *Alteromonas* in another sample (Redmond & Valentine, 2012). The oil sheen samples collected in June 2010 contained *Cyanobacteria* and *Alphaproteobacteria* (SAR11 clade, *Rhodobacterales*, and *Rhodospirillales*), with just 15% of sequences affiliated with possible hydrocarbon degraders from the *Alteromonadales* and *Oceanospirillales* (Redmond & Valentine, 2012). *Cycloclasticus* and *Colwellia* were minority

groups (< 5%) in the June oil slick samples (Redmond & Valentine, 2012). In another study, although *Alphaproteobacteria* and *Gammaproteobacteria* were still the dominant groups in the oil slick derived from May 2010, the community composition differed from the results obtained by Redmond and Valentine (Liu & Liu, 2013), indicating the slick samples were not homogeneous and their composition changed over time.

Large, mucous rich marine snow was observed floating at the surface in the immediate vicinity of oil layers near the wellhead area in May 2010; after one month, in late June, all GoM-snow had vanished from view (Passow *et al.*, 2012). Therefore, the rapid sedimentation of the oil marine snow was hypothesized since an *in situ* underwater camera caught videos of the sinking particles (Arne R. Diercks & Vernon Asper, ROV video, May 2010 R/V Pelican cruise, unpublished data). Scientists believed that as much as one third of the oil may have been entrained with deep currents at depths of more than 1000 meters, contaminated sediment as deep currents moved it around and dragged it to the bottom of the Gulf of Mexico, as so called “dirty blizzard” (Schrope, 2013). The estimated velocity of those sinking snow particles varies from 68 to 543 m/day (Passow *et al.*, 2012). Mixed together with the high nutrients, clay, minerals and low salinity river run-off from the Mississippi River and associated diversionary channels, the formation of oily marine snow was enhanced because of the stimulated phytoplankton growth by the nutrients and the increased snow aggregates by the mineral clays (MOSSFA-committee, 2013). Pyrogenic PAHs and soot particles derived from oil burning contributed to the formation of marine snow as well; however the contribution of these particles to the snow particles are still under investigation (MOSSFA-committee, 2013). Sediment trap data showed very high particulate organic carbon (POC) in August 2010 and decreasing thereafter. The lithogenic (i.e., silts and clays) component constituted 85% of the settled material in sediment trap; planktonic

inputs of carbonates and organic carbon and terrestrially organic matter composed the other significant portions (MOSSFA-committee, 2013). The degraded or partially degraded oil and the left-over heavy oil after weathering and biodegradation sink into the deep ocean is essential to transit oil from surface to the seafloor as organic carbon source (DOC or POC) (Ziervogel *et al.*, 2012).

Influence of the oil spill to the benthic environment

The “Dirty Blizzard” theory was established based on both the observation of the oily marine snow aggregates and the recovery of sediment cores with weathered oily particles on the top layer (Schrope, 2013). Lab incubation experiments generated marine snow aggregates that had high hydrolysis rates compare to the ambient seawater (Ziervogel *et al.*, 2012). Does it mean that the oil can be fully degraded before the oil marine snow particles make their way to the bottom? In the Oceanus cruise (21 August to 16 September , 2010), Joye et al. reported they found that potential oil-entrained sediment extended from close to the wellhead to as far as 37 miles southwest (Joye, 2011); on the top of the grey sediment, these sediment cores had an conspicuous red-brown surface layer inside which a lot of oil aggregates were found (Figure 7). Both visual and olfactory inspection indicated this top layer was contaminated by oil. In the Operational Science Advisory Team Report in 2010, oil - entrained sediments at a range of 3 km from the wellhead (mostly southwestern to the wellhead) were confirmed the DWH oil as their oil source (Zukunft, 2010) . Another cruise carried in December 2010 derived potentially oil contaminated sediment from northeast of the wellhead (Hollander *et al.*, 2013, Brooks *et al.*, 2014). More of the oil-entrained sediment had been recovered from near the wellhead area repeating from subsequent cruises. The analysis of the red-brown surface sediment by GC/FID and GC/MS indicated that 19 out of 64 samples within 5 km of the wellhead contained higher

concentration of the total petroleum hydrocarbon (TPH) than EPA standard (Mason *et al.*, 2014). These high TPH samples spread unevenly, without obvious geographical feature. The nitrogen concentration may explain their patchy distribution (Mason *et al.*, 2014). One of the factors contributed to the complexity of the oily snow precipitation could be the re-suspension of the surficial sediment caused by the deep current of the Gulf of Mexico, However, the hypothesis cannot be proved since there is no model to mimic the deep current yet.

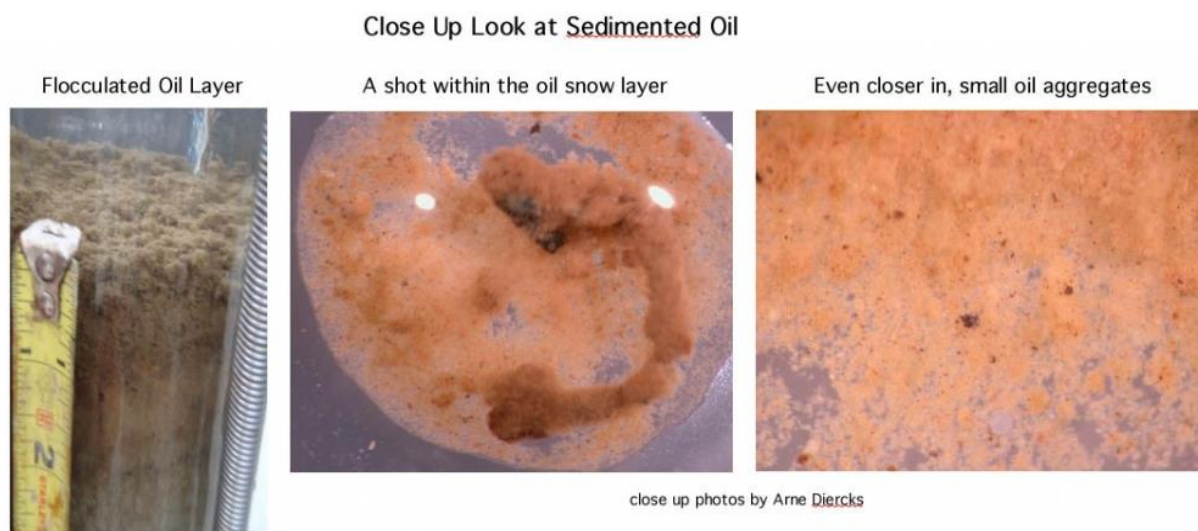


Figure 7. An obvious fluffy, red-brownish surface layer was found in September 2010 sediment cores collected close to the wellhead. A closer look of this layer revealed large amount of oil aggregates. (From Mandy Joye and Arne Diercks)

More support of the “Dirty Blizzard” theory was from isotopic data. Fast sedimentation rate was indicated by high activity of Th^{234} detected in the surficial sediment derived from 2010 (Brooks *et al.*, 2014). Th^{234} is an isotope of thorium, with its half-time as short as 24.5 days. The normal sedimentation rate is a long-term process which leads to depletion of Th^{234} in sediment. In another word, only fast sedimentation process could preserve high activity of Th^{234} . Coincidentally, high ^{14}C value were observed in the fluffy red-brown surficial sediment as well, manifested fast sedimentation happened to this surface layer in 2010 (Chanton, 2013). Excess

Th²³⁴ profiles reflect deposition of the 0.4-1.2 cm surface layer in a 4-5 month period in the late summer/fall of 2010, with higher mass accumulation rates (MARs) compared to average rates (past ~100 yrs). Using the same ²³⁴Th methodology, MARs have decreased over the two years following the event (Brooks *et al.*, 2014). The red-brown color was considered derived from two sediment layers enriched manganese oxide. Between the Mn maxima, a layer with a modest enrichment of Re, consistent with reducing sediments was sandwiched (Hastings *et al.*, 2014).

The reducing condition in the surficial sediment is generally considered driven by microorganisms, which maybe correspondently been affect as well. A study showed several *Alphaproteobacteria* and *Gammaproteobacteria* group (especially a *Colwellia* taxon), previously been found in the plume water during the spill appeared again in the polluted sediment (Mason *et al.*, 2014). Their metagenomic analysis indicated that nitrogen and hydrocarbons are the two main drivers to the community change in the polluted and non-polluted samples; the anaerobic process denitrification was very active as indicated by the annotated genes (Mason *et al.*, 2014). Analyzing the 3 representative sediment samples from Mason *et al.*'s collection by metagenomic sequencing, Kimes *et al.* found that two samples close to the wellhead contained high abundance of *Deltaproteobacteria*, especially the aromatic hydrocarbon degradation related anaerobic sulfate-reducing bacteria *Desulfobacterales*, *Desulfovibrionales* and *Desulfuromonadales* (Kimes *et al.*, 2013). The genes encoding aliphatic and simple aromatic hydrocarbons were most abundant in the contaminated sediment bacterial community, implying that the large amount of PAHs were recalcitrant to biodegradation and their persistence could have long-term impacts on the Gulf of Mexico seafloor (Mason *et al.*, 2014). In other study analyzing two sediments collected one year after the spill (Liu & Liu, 2013), however, had different dominant bacterial groups compare to the two studies above. In Liu & Liu's results, methanotrophs, *Pseudomonas*,

Vibrio, *Flavobacteria* and *Acidobacteria* were dominant in the 0–2-cm surface sediments. The differences between studies indicate that the oil impact to the seafloor was patchy and locally divergent; sediment bacterial community response to the oil input needs more detailed work.

The oil fallout seriously impacted the benthic macro-organisms as well. The redox condition change was the presumable reason for a community-wide decrease in benthic foraminifera (Hastings *et al.*, 2014). Reductions in abundance and diversity of the benthic macro-fauna was observed in a distance of 3 km to the wellhead, with moderate impacts detected to as far as 17 km to the southwest and 8.5 km to the northeast of the wellhead (MOSSFA-committee, 2013). These impacts correlated to the observed elevated TPH, PAH, and barium concentrations and the distance to wellhead (MOSSFA-committee, 2013). Deep sea coral suffered the oil fallout as well: at one site 13 km southwest of the wellhead, coral community covered by brown flocs presented wide spread signs of stress, including tissue loss, sclerite enlargement, excess mucous production and bleached commensal ophiuroids (White *et al.*, 2012). A more recent study found two other coral communities which were further away also been impacted by the spill (Fisher *et al.*, 2014). Other potential impact of the settled oil to deep sea fish and other organisms were also detected, as reported in the 2014 GOMRI meeting.

Oil transported to beach, salt marshes

Gulf of Mexico coastal habitats generates more than \$10 billion per year in revenues by fisheries and tourism (Silliman *et al.*, 2012). However, approximately $9 \pm 4 \times 10^7$ kg of the surface oil was transported to the beaches and salt marshes shoreline of Alabama, Louisiana, Florida and Mississippi, polluted ~ 1,100 miles of shoreline (Ramseur & Hagerty, 2014). The beaches were closed in early June, as of December 2012, 339 miles of coastline remain subject to evaluation and/or cleanup operations (Ramseur & Hagerty, 2014), largely damaged the fisheries

and tourism. Partially weathered tar balls and oil aggregates were found on the beaches starting in June, and were buried to depth by newly arrived sands in a short time. For example, the tarballs and tar mats began washing ashore around June 23 in Pensacola beach; in early July, oil patties and tarballs were discovered as deep as 0.6 m (National Geographic report, July 2, 2010). The sand patty and rock scrapping collected from oiled beaches between July 2010 and November 2011 showed extensive biodegradation, as indicated by much less resolvable alkanes, and lower n-C18/phytane values than the sea surface oil slick from June 2010 (2.1 vs. 2.5) (Aeppli *et al.*, 2012). The total concentration of 30 PAHs decreased further from 7.7-13 mg g⁻¹ oil for surface slicks, to 0.6-2.8 mg g⁻¹ extractable material for sand patties and rock scrapings (Aeppli *et al.*, 2012). PAH losses calculated by normalization to hopane were 83-98% for sand patties and 93% for the rock scraping (Aeppli *et al.*, 2012). In the meanwhile, the operationally defined oxygenated fractions increased significantly in sand patties and rock scrapings, another evidence for intensive biodegradation (Aeppli *et al.*, 2012). Microbial community survey showed bloom of bacterial abundance (predominately by Gammaproteobacteria and Alphaproteobacteria) in oiled sands, especially the alkane and aliphatic compounds degrader *Alcanivorax* and *Marinobacter* (Kostka *et al.*, 2011). Thanks to the vegetation from the marsh edge, only the outer marsh regions were impacted by oil (within 15 m from the marsh edge), where levels of total PAHs in the polluted sediment was >100 times higher than intact sediment. However, high portion of plant in this region were dead (36%-95%) (Silliman *et al.*, 2012). Heavier fractions were resistant in marsh sediments, contrast to the lighter hydrocarbons that were rapidly degraded by known hydrocarbon degrading bacteria such as *Rhodobacterales* and *Sphingomonadales*, as well as high abundance of sulfate reducing bacteria coincident with the high level sulfite concentration (Natter *et al.*, 2012). There is evidence showing oil in the water column entered the

food web as carbon, however, more careful studies are required before connect the oil to the spill oil from the Macondo wellhead (Chanton, 2013).

As demonstrated above, study the Gulf of Mexico oil spill and its effect to the ecosystem is a hard task, since the residue oil interacts with various environments not only tightly influence each other but also has complex features individually. Because microbial mediated biodegradation is the only way to transform oil-derived hydrocarbons into biomass or remineralize them to CO₂, an essential part to understand the fate of the released oil is to study the dynamics of microorganisms in the oil polluted environments. My dissertation focuses on the microbial community structure affected by the released oil both in water column and in seafloor sediment, trying to understand the successional change of microbial community and establish hypothesis for the biodegradation occurred throughout the spill event by this long term study based on the time series samples taken before, during and after the oil spill, both from water column and sediment.

CHAPTER 1: PULSED BLOOMS AND PERSISTENT OIL-DEGRADING BACTERIAL POPULATIONS IN THE WATER COLUMN DURING AND AFTER THE DEEPWATER HORIZON BLOWOUT¹

1.1 Abstract

One of the defining features of the Deepwater Horizon oil spill was the rapid formation and persistence of a hydrocarbon plume in deep water. Here we use 16S rRNA gene clone libraries and pyrosequencing of 16S rRNA gene fragments to outline the temporal dynamics of the bacterial community in the water column near the Macondo wellhead. Our timeline starts with the pre-spill (March 2010) status of the water column bacterial community, continues through the bacterial enrichments dominating the hydrocarbon plume after the blowout (DWH *Oceanospirillales*, *Cycloclasticus*, *Colwellia*) in late May 2010), and leads towards post-spill bacterial communities with molecular signatures related to degradation of phytoplankton pulses (September and October 2010; July 2011) in the water column near the Macondo wellhead. We document a dramatic transition as the complex bacterial community before the oil spill was temporarily overwhelmed by a few specialized bacterial groups responding to the massive influx of hydrocarbons in May 2010. In September and October 2010, this bacterial bloom had been replaced by a diversified bacterial community which resembled its predecessor prior to the spill.

¹ This chapter was previously published as an article in *Deep Sea Research Part II: Topical Studies in Oceanography*. The original citation is as follow: Tingting Yang, Lisa M. Nigro, Tony Gutierrez, Lindsay D'Ambrosio, Samantha B. Joye, Raymond Highsmith, Andreas Teske, Pulsed blooms and persistent oil-degrading bacterial populations in the water column during and after the Deepwater Horizon blowout, *Deep Sea Research Part II: Topical Studies in Oceanography*, Available online 23 January 2014, ISSN 0967-0645, <http://dx.doi.org/10.1016/j.dsr2.2014.01.014>.

Notably, the post-plume 16S rRNA gene clone libraries and pyrosequencing datasets illustrated the continued presence of oil-degrading bacteria in the water column near the Macondo wellhead which we posit to represent an inherent signature of hydrocarbon catabolic potential to the Gulf of Mexico. The pyrosequencing results detected and tracked minority bacterial populations that were not visible in the conventional 16S rRNA gene clone libraries and allowed us to identify natural reservoirs of the Deepwater Horizon *Oceanospirillales* within and outside of the Gulf of Mexico.

1.2 Introduction

The explosion and sinking of the Deepwater Horizon platform discharged oil and gas into the Gulf of Mexico and generated massive and long-lasting perturbations in its ecosystem (Schrope 2011). One of the defining features of the Deepwater Horizon oil spill was the formation of a deepwater hydrocarbon-enriched plume during the multiphase ejection of gas and oil from the wellhead. The plume was positioned between approx. 1000 and 1300 m depth due to preferential entrainment of the soluble complex hydrocarbons within the deep, cold (5 °C) water, and consisted mostly of light alkanes (C₁ to C₃), BTEX, submicrometer-size oil droplets (Ryerson et al. 2012, Reddy et al. 2012); it also entrained the dispersant compound dioctyl sodium sulfosuccinate (DOSS) (Kujawinski et al. 2011). The deep plume was detected initially in early May 2010 (Diercks et al. 2010b), and its gradual spread was monitored throughout the summer of 2010 (Hazen et al. 2010, Camilli et al. 2010, Kessler et al. 2011, Joye et al. 2011b) by tracking local oxygen depletion and C-DOM fluorescence maxima as proxies for the presence of hydrocarbons and microbial activity (Diercks et al. 2010b, Wade et al. 2011). However, tracking the evolving composition of the bacterial community in the oil-impacted water column, including the deep hydrocarbon plume, during 2010 was an extraordinary challenge.

Initially, changes of the microbial community in the water column were inferred from Phylochip® analyses of oil degrading communities (Hazen et al. 2010), or from models of methane, ethane and propane dynamics (Valentine et al. 2010, Kessler et al. 2011). These studies did not provide exact information on sampling times, water depths and geographical positions for their molecular data. Additional 16S rRNA gene clone library datasets were recently synthesized and published with precise sampling locations and times, in order to coherently survey the changing bacterial community composition over the lifetime of the deep hydrocarbon plume (Redmond and Valentine 2012). In late May 2010, the plume-associated bacterial community was dominated by a specific cluster within the *Oceanospirillales*, subsequently termed Deep Water Horizon (DWH) *Oceanospirillales*, before changing in mid-June to a community where most clones grouped with the genera *Cycloclasticus*, obligate degraders of aromatic hydrocarbons, and *Colwellia*, known as a genus of psychrophilic marine heterotrophic generalists. By early September, the bacterial community had diversified considerably and included different *Alphaproteobacteria*, multiple lineages within the *Gammaproteobacteria*, *Flavobacteria*, and several other phylum-level lineages such as the *Actinobacteria*, *Planctomycetes*, *Chloroflexi*, and the SAR406 cluster (Redmond and Valentine 2012).

Here we extend the timeline of microbial oil spill response with molecular analyses of samples from March 2010 to July 2011 (Table 1). By complementing clone libraries of nearly full-length 16S rRNA genes with pyrosequencing surveys of shorter 16S rRNA gene fragments, we combine the taxonomic precision of full-length 16S rRNA genes with the high-throughout resolution of bacterial community structure enabled by pyrosequencing. Specifically, we extend previous molecular analyses in three ways. 1) The pre-spill (March 10, 2010) water column bacterial community is compared to post-spill communities (September 12 and October 18,

2010; July 3, 2011) near the Macondo wellhead with 16S rRNA gene clone libraries. 2) A water column profile near the Macondo wellhead with samples above, within and below the deep hydrocarbon plume during its *Oceanospirillales*-dominated phase (May 31, 2010) is analyzed with conventional 16S rRNA gene clone libraries and by 16S rRNA gene fragment pyrosequencing. 3) The water column profile is compared to surface water samples contaminated with weathered oil from early May 2010 (May 5, 2010), and post-plume water samples (September 12 and October 18, 2010) from near the wellhead and east of the wellhead, using pyrosequencing.

1.3 Material and Methods

1.3.1 Samples and the molecular process.

Sampling. Surface and water column samples were obtained during six research cruises (Table 1). The pre-spill sample (March 10, 2010) was obtained on RV *Pelican* by CTD cast at 800 m depth, ca. 10 nautical miles northwest of the Macondo wellhead (28 °50.43 N, 88 °30.29 W). The water column did not show any of oxygen or CDOM anomalies. From May 5 to 9, Oil spill surface water samples were collected via bucket sampling from the R/V *Pelican*, and kept at ca. 4 °C during and after immediate transport to Chapel Hill. Surface water sampled ca. 0.5 nautical miles from the wellhead (28 °44.175 N, 88 °22.335 W, May 5, 2010) showed the strongest admixture of reddish-brown weathered oil sludge, and was used for DNA sequencing. These surface seawater samples are to the best of our knowledge the first samples collected on the earliest Rapid Response cruise to the Deepwater Horizon response zone (May 5 to 9, 2010; Diercks et al. 2010a). CTD surveys during the second cruise leg (May 10 to 16, 2010) provided the first evidence of the southwest-trending hydrocarbon plume in the deep water column (Diercks et al., 2010b). About three weeks later, a water column profile with four depths bracketing the deepwater plume was obtained by CTD approx. 4.7 nautical miles southwest of

the wellhead (R/V *Walton Smith*, May 31, 2010; 28 °41.686 N, 88 °26.081 W). Water samples of approx. 500 ml were collected at 800, 1170, 1210, and 1320 m depth. Immediately after shipboard recovery, they were filtered through 47 mm diameter and 0.22 µm poresize Anodisc filters; the filters were placed on dry ice until DNA extraction in Chapel Hill. The 1170 m and the 1210 m samples of this profile represent the deepwater hydrocarbon plume, as indicated by localized oxygen depletion and increased water column fluorescence measured during the CTD cast. On September 12, almost two months after the Macondo wellhead had been capped on July 15, 2010, water column filter samples were collected again at the same location (R/V *Pelican*; 28 °41.713 N, 88 °26.073 W) to evaluate the water column bacterial community at 800 and 1210 m depth (Postplume I). CTD profiles no longer detected the *in-situ* indicators (localized oxygen depletion coinciding with fluorescence maximum) of the deep hydrocarbon plume, consistent with the deepwater circulation of the Gulf of Mexico that moved the deep hydrocarbon plume in a southwesterly direction already at the onset of the spill (Diercks et al. 2010b). A negative control sample (Postplume II) was obtained 37 nautical miles east of the wellhead (28 °40.503 N, 87 °39.250 W) at a depth of 1052 m (R/V *Cape Hatteras*, October 18, 2010). Due to the predominantly west and southwest deepwater current pattern in this area, this sample was unlikely to have been in contact with the Macondo wellhead and any residual hydrocarbon leakage at this location. In July 2011, the water column near the Macondo wellhead was sampled again (July 3; R/V *Endeavor*; 28 °42.177 N, 88 °21.240 W), to initiate a multiannual survey of water column microbial community structure (Postplume III).

Sample names with cruise-specific sampling codes in parentheses	Ship	Date	Depth (m)	Latitude (N)	Longitude (W)
Prespill-800m	RV <i>Pelican</i>	March 10, 2010	800	28°50.43	88°30.29
SurfaceOil-PE5	RV <i>Pelican</i>	May 5, 2010	0	28°44.175	88°22.335
Plumeprofile-800m (B11)	RV <i>Walton Smith</i>	May 31, 2010	800	28°41.686	88°26.081
Plumeprofile-1170m (B6)	RV <i>Walton Smith</i>	May 31, 2010	1170	28°41.686	88°26.081
Plumeprofile-1210m (B3)	RV <i>Walton Smith</i>	May 31, 2010	1210	28°41.686	88°26.081
Plumeprofile-1320m (B1)	RV <i>Walton Smith</i>	May 31, 2010	1320	28°41.686	88°26.081
Postplume I-800m (C4B8)	RV <i>Pelican</i>	Sept 12, 2010	800	28°41.713	88°26.073
Postplume I-1210 m (C4B4)	RV <i>Pelican</i>	Sept 12, 2010	1210	28°41.713	88°26.073
Postplume II (GIP22)	RV <i>Cape Hatteras</i>	Oct 18, 2010	1052	28°40.503	87°39.250
Postplume III (E002)	RV <i>Endeavor</i>	July 3, 2011	1100	28°42.177	88°21.240

Table 1. Samples collected on multiple research cruises near the Macondo wellhead with dates, water depths, and geographical coordinates.

DNA Extraction. DNA from sediment was extracted and purified from water sample filters as described previously (Teske et al. 2011). Using sterile forceps, one quarter of each frozen filter was placed and crushed into a 2 ml microcentrifuge tube containing sterile 200 μ L of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5), with 0.5 % (wt/vol) sodium dodecyl sulfate and proteinase K (50 μ g mL⁻¹). Reactions were incubated at room temperature for 30 minutes followed by the addition of 200 μ L equilibrated phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) and vortexing (5 min) at room temperature. The aqueous phase was separated from the phenol phase by centrifugation at 10,000 x g for 5 minutes, and transferred into a new microcentrifuge tube. The organic phase was re-extracted with another 200 μ L volume of sterile TE (pH 7.5), followed by incubation for 5 minutes at room temperature and phase separation by centrifugation for 5 minutes. From the combined aqueous phases, DNA was precipitated by adding 10% (v/v) 5 M sodium chloride and 2.5 volume of 100% ethanol. Samples were then centrifuged at 13,000 x g for 15 minutes at 4 °C. The pellets were washed with 70% ethanol and centrifuged for 15 minutes at 10,000 x g at 4 °C. The ethanol was decanted and the pellets were air dried before resuspension in 50 μ L of sterile TE.

PCR Amplification and Cloning. The bacterial 16S rRNA gene was amplified with Speedstar DNA polymerase (TaKaRa, Shiga, Japan) using the bacterial primers 8f and 1492r (Teske et al. 2002) and the manufacturer's recommended concentration for buffer, dNTPs and DNA polymerase. Each PCR reaction consisted of 2 µl DNA extract, 2.5 µl 10X FBI buffer (TaKaRa, Shiga, Japan), 2.0 µl dNTP mix, 2.0 µl 10 µM solution of primers 8F and 1492R, respectively (both primers from Invitrogen, Carlsbad, CA), and 0.25 µl SpeedStar polymerase (TaKaRa), and was brought to 25 µl with sterile H₂O. Amplification was performed in a BioRad iCycler Thermal Cycler (Hercules, CA) as follows: initial denaturation at 95 °C for 4 minutes, 25 cycles of 95 °C (10 seconds), 55 °C (15 seconds) and 72 °C (20 seconds), and a final 10 minute extension of 72 °C. PCR product aliquots, including positive and negative controls, were SYBR green stained and visualized using a 1.5% agarose gel. The PCR products were purified using the MinElute® PCR Purification Kit according to the manufacturer's instructions (Qiagen, Valencia, CA), and cloned into OneShot® TOP10 competent Cells (Invitrogen, Carlsbad, CA) using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Transformed cells were grown on LB/Xgal/Kanamycin plates. Individual white colonies were arbitrarily picked, re-plated and sanger-sequenced at Genewiz Corporation (South Plainfield, NJ) using vector primers M13 F and M13 R.

1.3.2. Phylogenetic Analysis.

Near-complete 16S rRNA gene sequences were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) and compared to other sequences via the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/>) (Altschul et al. 1990). After construction of a general 16S rRNA alignment using the ARB phylogeny software package (Ludwig et al. 2004) and the SILVA v95 database (Pruesse et al. 2007),

separate alignments for the *Gamma*- and *Alphaproteobacteria* were prepared with sequences for related *Gammaproteobacteria* and *Alphaproteobacteria*. Sequences of well-characterized pure cultures and described species were used for phylogenies whenever possible; otherwise, molecular phylotypes with an informative literature history were selected to anchor major phylogenetic branches of uncultured bacteria. Phylogenetic trees were constructed and bootstrap checks (1000 reruns) of the tree topology were performed using ARB's neighbor-joining function with Jukes-Cantor correction. Sequences were deposited at NCBI Genbank with accession numbers JN015198 to JN015212 and JX878917 to JX879086 (Table 2).

Genbank #	Sample name	Genbank #	Sample name	Genbank #	Sample name
JX878975	Postplume-II_1050m_050	JX879018	Prespill-800m-020	JX879061	Postplume-III-1100m-049
JX878976	Postplume-II_1050m_064	JX879019	Prespill-800m-021	JX879062	Postplume-III-1100m-050
JX878977	Postplume-II_1050m_003	JX879020	Prespill-800m-023	JX879063	Postplume-III-1100m-057
JX878978	Postplume-II_1050m_005	JX879021	Prespill-800m-029	JX879064	Postplume-III-1100m-059
JX878979	Postplume-II_1050m_013	JX879022	Prespill-800m-030	JX879065	Postplume-III-1100m-060
JX878980	Postplume-II_1050m_016	JX879023	Prespill-800m-034	JX879066	Postplume-III-1100m-061
JX878981	Postplume-II_1050m_019	JX879024	Prespill-800m-038	JX879067	Postplume-III-1100m-062
JX878982	Postplume-II_1050m_021	JX879025	Prespill-800m-041	JX879068	Postplume-III-1100m-064
JX878983	Postplume-II_1050m_024	JX879026	Prespill-800m-048	JX879069	Postplume-III-1100m-065
JX878984	Postplume-II_1050m_071	JX879027	Prespill-800m-055	JX879070	Postplume-III-1100m-068
JX878985	Postplume-II_1050m_078	JX879028	Prespill-800m-062	JX879071	Postplume-III-1100m-069
JX878986	Postplume-II_1050m_084	JX879029	Prespill-800m-063	JX879072	Postplume-III-1100m-070
JX878987	Postplume-I-800m-001	JX879030	Prespill-800m-064	JX879073	Postplume-III-1100m-073
JX878988	Postplume-I-800m-007	JX879031	Prespill-800m-065	JX879074	Postplume-III-1100m-074
JX878989	Postplume-I-800m-011	JX879032	Prespill-800m-066	JX879075	Postplume-III-1100m-078
JX878990	Postplume-I-800m-012	JX879033	Prespill-800m-068	JX879076	Postplume-III-1100m-079
JX878991	Postplume-I-800m-016	JX879034	Prespill-800m-069	JX879077	Postplume-III-1100m-080
JX878992	Postplume-I-800m-022	JX879035	Prespill-800m-073	JX879078	Postplume-III-1100m-081
JX878993	Postplume-I-800m-032	JX879036	Prespill-800m-074	JX879079	Postplume-III-1100m-085
JX878994	Postplume-I-800m-033	JX879037	Prespill-800m-085	JX879080	Postplume-III-1100m-086
JX878995	Postplume-I-800m-034	JX879038	Prespill-800m-086	JX879081	Postplume-III-1100m-088
JX878996	Postplume-I-800m-036	JX879039	Prespill-800m-092	JX879082	Postplume-III-1100m-090
JX878997	Postplume-I-800m-039	JX879040	Prespill-800m-093	JX879083	Postplume-III-1100m-091
JX878998	Postplume-I-800m-040	JX879041	Postplume-III-1100m-001	JX879084	Postplume-III-1100m-095
JX878999	Postplume-I-800m-047	JX879042	Postplume-III-1100m-003	JX879085	Postplume-II_1050m_062
JX879000	Postplume-I-800m-048	JX879043	Postplume-III-1100m-008	JX879086	Postplume-II_1050m_076
JX879001	Postplume-I-800m-052	JX879044	Postplume-III-1100m-010	JN015198	Plumeprofile_1320m_229
JX879002	Postplume-I-800m-057	JX879045	Postplume-III-1100m-011	JN015199	Plumeprofile_1320m_254
JX879003	Postplume-I-800m-059	JX879046	Postplume-III-1100m-013	JN015200	Plumeprofile_1320m_272
JX879004	Postplume-I-800m-060	JX879047	Postplume-III-1100m-014	JN015201	Plumeprofile_1320m_277
JX879005	Postplume-I-800m-068	JX879048	Postplume-III-1100m-015	JN015202	Plumeprofile_800m_188
JX879006	Postplume-I-800m-069	JX879049	Postplume-III-1100m-018	JN015203	Plumeprofile_800m_208
JX879007	Postplume-I-800m-071	JX879050	Postplume-III-1100m-020	JN015204	Plumeprofile_800m_001
JX879008	Postplume-I-800m-072	JX879051	Postplume-III-1100m-022	JN015205	Plumeprofile_800m_013
JX879009	Postplume-I-800m-074	JX879052	Postplume-III-1100m-024	JN015206	Plumeprofile_800m_029
JX879010	Postplume-I-800m-089	JX879053	Postplume-III-1100m-029	JN015207	Plumeprofile_1320m_035
JX879011	Postplume-I-800m-095	JX879054	Postplume-III-1100m-030	JN015208	Plumeprofile_1210m_039
JX879012	Prespill-800m-008	JX879055	Postplume-III-1100m-031	JN015209	Plumeprofile_1210m_053
JX879013	Prespill-800m-009	JX879056	Postplume-III-1100m-033	JN015210	Plumeprofile_1210m_076
JX879014	Prespill-800m-010	JX879057	Postplume-III-1100m-034	JN015211	Plumeprofile_1210m_100
JX879015	Prespill-800m-011	JX879058	Postplume-III-1100m-035	JN015212	Plumeprofile_1170m_038
JX879016	Prespill-800m-013	JX879059	Postplume-III-1100m-038		
JX879017	Prespill-800m-014	JX879060	Postplume-III-1100m-042		

Table 2. Genbank numbers for 16S rRNA gene clones.

1.3.3. Pyrosequencing of partial 16S rRNA gene sequences.

Highly variable portions of 16S rRNA genes (*E.coli* positions 28 to 337) were amplified with five barcoded bacterial 16S-targeted primer pairs (Table 3) to generate ca. 300 bp-long PCR

products. The PCR products were purified using MiniElute PCR Purification kit (QIAGEN)

Primer set	Name	Primer
Set 1:	AB1_27F (Forward primer)	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACAGTGCAGAGTTTGATCCTGGCTCAG-3'
	AB1_338R (Reverse primer)	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCACAGTGCATGCTGCCTCCCGTAGGAGT-3'
Set 2:	AB1_27F (Forward primer)	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACGAGTTCAGAGTTTGATCCTGGCTCAG-3'
	AB1_338R (Reverse primer)	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCACGAGTGCATGCTGCCTCCCGTAGGAGT-3'
Set 3:	AB1_27F (Forward primer)	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCACTCACTCAGAGTTTGATCCTGGCTCAG-3'
	AB1_338R (Reverse primer)	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCACTCACCATGCTGCCTCCCGTAGGAGT-3'
Set 4:	AB1_27F (Forward primer)	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGACACTCAGAGTTTGATCCTGGCTCAG-3'
	AB1_338R (Reverse primer)	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCAGACACCATGCTGCCTCCCGTAGGAGT-3'
Set 5:	AB1_27F (Forward primer)	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCAGCACATCAGAGTTTGATCCTGGCTCAG-3'
	AB1_338R (Reverse primer)	5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTCAGCACACATGCTGCCTCCCGTAGGAGT-3'

Table 3: Barcoded PCR Primer sets for 16S rRNA pyrosequencing.

and stored in 1 × TE buffer for pyrosequencing analysis using the Roche 454 GS LFX Titanium Sequencer in the Microbiome Core Facility at the University of North Carolina at Chapel Hill (www.med.unc.edu/microbiome). Raw data were trimmed and filtered using LUCY to remove poor quality reads (minimum PHRED score of 27.5) and those of less than 200 nt (Kunin *et al.*, 2010). The 8 nt barcode was used to de-multiplex and assign reads to samples using QIIME (Caporaso *et al.*, 2010). The reads were binned into operational taxonomic units (OTUs) at 97% sequence identity with UCLUST (Edgar, 2010) followed by selection of a representative sequence based on the most abundant unique read within each cluster. After initial phylum- and family-level identification using BLAST, the 300-bp fragments were imported and aligned into ARB, using the previously prepared full-length 16S rRNA gene alignments of the water column sequences, and related published sequences, as templates. In addition, the gamma- and alphaproteobacterial alignments were manually edited, and >90% of all pyrosequencing fragments could be assigned

to genus- or family-level phylogenetic branches defined by 16S rRNA gene clone library sequences (Table 4).

OTUs		PLP-1320m		PLP-1210m		PLP-1170m		PLP-800m		POP-I-1210m		POP-I-800m		POP-II-1050m		Surfaceoil-PE5
Gamma_DWH_plume_group	122	5.69%	373	90.53%	709	69.37%	0	0.00%	17	0.20%	0	0.00%	2	0.04%	9	0.04%
Gamma_DWH_plume_related	3	0.14%	0	0.00%	7	0.68%	0	0.00%	1	0.01%	2	0.02%	1	0.02%	0	0.00%
Gamma_Cycloclasticus	63	2.94%	27	6.55%	135	13.21%	1	0.79%	447	5.29%	21	0.19%	43	0.85%	22336	93.07%
Gamma_Cycloclasticus_related	5	0.23%	0	0.00%	12	1.17%	0	0.00%	35	0.41%	2	0.02%	3	0.06%	564	2.35%
Gamma_Methylotrophs_and_relateds	12	0.56%	0	0.00%	0	0.00%	0	0.00%	400	4.74%	25	0.22%	69	1.36%	0	0.00%
Gamma_Colwellia	0	0.00%	4	0.97%	31	3.03%	0	0.00%	6	0.07%	5	0.04%	2	0.04%	41	0.17%
Gamma_Colwellia_related	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	8	0.03%
Gamma_Acinetobacter	12	0.56%	0	0.00%	0	0.00%	0	0.00%	1	0.01%	2	0.02%	9	0.18%	0	0.00%
Gamma_Agarivorans	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	2	0.01%
Gamma_AGG47related1	8	0.37%	0	0.00%	0	0.00%	1	0.79%	34	0.40%	94	0.84%	56	1.10%	0	0.00%
Gamma_AGG47related2	30	1.40%	0	0.00%	1	0.10%	5	3.97%	41	0.49%	131	1.16%	52	1.03%	1	0.00%
Gamma_Alcanivorax	0	0.00%	1	0.24%	0	0.00%	0	0.00%	14	0.17%	14	0.12%	14	0.28%	1	0.00%
Gamma_Alteromonadales	0	0.00%	0	0.00%	0	0.00%	1	0.79%	1	0.01%	0	0.00%	3	0.06%	41	0.17%
Gamma_Alteromonas	0	0.00%	0	0.00%	0	0.00%	0	0.00%	184	2.18%	166	1.48%	10	0.20%	349	1.45%
Gamma_Arctic96BD19	80	3.73%	0	0.00%	6	0.59%	3	2.38%	307	3.64%	557	4.95%	140	2.76%	0	0.00%
Gamma_Congregibacter_and_relateds	10	0.47%	0	0.00%	0	0.00%	0	0.00%	115	1.36%	100	0.89%	36	0.71%	13	0.05%
Gamma_Fangia_related	15	0.70%	0	0.00%	0	0.00%	0	0.00%	40	0.47%	12	0.11%	17	0.34%	0	0.00%
Gamma_Halomonas	0	0.00%	0	0.00%	0	0.00%	0	0.00%	7	0.08%	5	0.04%	2	0.04%	33	0.14%
Gamma_Idiomarina	0	0.00%	0	0.00%	0	0.00%	0	0.00%	32	0.38%	29	0.26%	0	0.00%	0	0.00%
Gamma_Legionellales	0	0.00%	0	0.00%	0	0.00%	2	1.59%	16	0.19%	11	0.10%	8	0.16%	0	0.00%
Gamma_Marinimicrobium	1	0.05%	0	0.00%	0	0.00%	0	0.00%	11	0.13%	19	0.17%	18	0.35%	0	0.00%
Gamma_Marinobacter	0	0.00%	0	0.00%	1	0.79%	1	0.79%	17	0.20%	79	0.70%	5	0.10%	5	0.02%
Gamma_Marinomonas	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	0.02%	0	0.00%
Gamma_Methylophaga	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	0.01%	5	0.10%	0	0.00%
Gamma_Methylophaga_sistergroup	0	0.00%	0	0.00%	0	0.00%	0	0.00%	171	2.03%	0	0.00%	30	0.59%	0	0.00%
Gamma_Moritella	7	0.33%	0	0.00%	0	0.00%	0	0.00%	1	0.01%	1	0.01%	2	0.04%	1	0.00%
Gamma_Neptunomonas	0	0.00%	0	0.00%	0	0.00%	0	0.00%	1	0.01%	1	0.01%	3	0.06%	0	0.00%
Gamma_Oceanobacter	0	0.00%	0	0.00%	0	0.00%	0	0.00%	59	0.70%	135	1.20%	0	0.00%	0	0.00%
Gamma_Oceanospirillales	0	0.00%	0	0.00%	0	0.00%	0	0.00%	125	1.48%	4	0.04%	9	0.18%	7	0.03%
Gamma_Oceanospirillum	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Gamma_Oleiphilus	8	0.37%	0	0.00%	1	0.10%	0	0.00%	74	0.88%	53	0.47%	16	0.32%	0	0.00%
Gamma_Oleispira	63	2.94%	0	0.00%	3	0.29%	0	0.00%	1	0.01%	0	0.00%	0	0.00%	7	0.03%
Gamma_Pseudoalteromonas	0	0.00%	0	0.00%	0	0.00%	0	0.00%	9	0.11%	11	0.10%	2	0.04%	287	1.20%
Gamma_Pseudomonas	41	1.91%	0	0.00%	0	0.00%	0	0.00%	2	0.02%	5	0.04%	24	0.47%	0	0.00%
Gamma_SAR156	10	0.47%	0	0.00%	0	0.00%	0	0.00%	7	0.08%	23	0.20%	7	0.14%	0	0.00%
Gamma_SAR86	0	0.00%	0	0.00%	0	0.00%	0	0.00%	2	0.02%	1	0.01%	0	0.00%	4	0.02%
Gamma_Shewanella	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Gamma_Stenotrophomonas	0	0.00%	0	0.00%	0	0.00%	7	5.56%	0	0.00%	0	0.00%	2	0.04%	0	0.00%
Gamma_SUP05_and_relateds	86	4.01%	0	0.00%	0	0.00%	0	0.00%	86	1.02%	19	0.17%	17	0.34%	0	0.00%
Gamma_SUP05_Arctic_related	77	3.59%	0	0.00%	4	0.39%	0	0.00%	4	0.05%	4	0.02%	0	0.00%	1	0.00%
Gamma_Unidentified	0	0.00%	1	0.24%	0	0.00%	0	0.00%	21	0.25%	41	0.36%	36	0.71%	13	0.05%
Gamma_Vibrionales	4	0.19%	0	0.00%	0	0.00%	0	0.00%	5	0.06%	4	0.04%	3	0.06%	5	0.02%
Gamma_ZD0417	1	0.05%	0	0.00%	1	0.10%	0	0.00%	9	0.11%	6	0.05%	2	0.04%	1	0.00%
Gamma_ZD0417_related	56	2.61%	0	0.00%	5	0.49%	3	2.38%	159	1.88%	229	2.04%	172	3.39%	0	0.00%
Alpha_Bradyrhizobium	0	0.00%	1	0.24%	0	0.00%	0	0.00%	1	0.01%	5	0.04%	12	0.24%	0	0.00%
Alpha_clusterA	48	2.24%	0	0.00%	0	0.00%	1	0.79%	68	0.81%	95	0.84%	50	0.99%	0	0.00%
Alpha_clusterB	5	0.23%	0	0.00%	1	0.10%	0	0.00%	24	0.28%	34	0.30%	22	0.43%	0	0.00%
Alpha_Magnetospirillum	0	0.00%	0	0.00%	0	0.00%	0	0.00%	5	0.06%	3	0.03%	2	0.04%	0	0.00%
Alpha_Oceanibaculum	23	1.07%	0	0.00%	0	0.00%	4	3.17%	23	0.27%	26	0.23%	18	0.35%	0	0.00%
Alpha_Rhizobiales	1	0.05%	0	0.00%	0	0.00%	0	0.00%	16	0.19%	10	0.09%	40	0.79%	0	0.00%
Alpha_Rhodoplanes	1	0.05%	0	0.00%	0	0.00%	0	0.00%	5	0.06%	2	0.02%	2	0.04%	0	0.00%
Alpha_Rhodospirillales	10	0.47%	0	0.00%	0	0.00%	0	0.00%	77	0.91%	88	0.78%	61	1.20%	2	0.01%
Alpha_Roseobacter	15	0.70%	0	0.00%	0	0.00%	0	0.00%	25	0.30%	11	0.10%	4	0.08%	28	0.12%
Alpha_SAR11_pelagibacter_related	0	0.00%	0	0.00%	1	0.10%	1	0.79%	47	0.56%	114	1.01%	40	0.79%	8	0.03%
Alpha_SAR11_ING	38	1.77%	0	0.00%	3	0.29%	2	1.59%	168	1.99%	408	3.63%	180	3.55%	0	0.00%
Alpha_SAR11_SAR193	55	2.57%	0	0.00%	6	0.59%	1	0.79%	141	1.67%	592	5.26%	151	2.98%	0	0.00%
Alpha_SAR11_SAR203	2	0.09%	0	0.00%	2	0.20%	3	2.38%	13	0.15%	67	0.60%	7	0.14%	0	0.00%
Alpha_SAR11_SAR220	39	1.82%	0	0.00%	3	0.29%	0	0.00%	92	1.09%	274	2.44%	112	2.21%	0	0.00%
Alpha_SAR11_SAR241	23	1.07%	0	0.00%	3	0.29%	1	0.79%	103	1.22%	392	3.48%	142	2.80%	0	0.00%
Alpha_SAR11_SAR407	26	1.21%	0	0.00%	5	0.49%	1	0.79%	99	1.17%	331	2.94%	109	2.15%	14	0.06%
Alpha_SAR11_SAR464	13	0.61%	0	0.00%	7	0.68%	0	0.00%	52	0.62%	213	1.89%	82	1.62%	0	0.00%
Alpha_SAR11_unnamed	41	1.91%	1	0.24%	4	0.39%	4	3.17%	138	1.63%	526	4.68%	206	4.06%	0	0.00%
Alpha_Sphingomonadales	21	0.98%	1	0.24%	0	0.00%	11	8.73%	106	1.26%	124	1.10%	269	5.30%	0	0.00%
Alpha_unidentified	17	0.79%	0	0.00%	1	0.10%	0	0.00%	25	0.30%	44	0.39%	47	0.93%	2	0.01%
Acidobacteria	30	1.40%	1	0.24%	1	0.10%	4	3.17%	53	0.63%	104	0.92%	88	1.73%	0	0.00%
Actinobacteria	34	1.59%	1	0.24%	0	0.00%	6	4.76%	137	1.62%	300	2.67%	144	2.84%	4	0.02%
Bacteroidetes	81	3.78%	0	0.00%	2	0.20%	4	3.17%	570	6.75%	577	5.13%	185	3.65%	104	0.43%
Chloroflexi	20	0.93%	1	0.24%	6	0.59%	1	0.79%	211	2.50%	291	2.59%	111	2.19%	0	0.00%
Cyanobacteria	2	0.09%	0	0.00%	0	0.00%	7	5.56%	46	0.54%	100	0.89%	39	0.77%	73	0.30%
Delta_SAR324	197	9.19%	0	0.00%	14	1.37%	25	19.84	646	7.65%	1226	10.90%	511	10.07%	0	0.00%
Delta_Nitrospina	36	1.68%	0	0.00%	2	0.20%	1	0.79%	125	1.48%	327	2.91%	138	2.72%	0	0.00%
Delta_others	66	3.08%	0	0.00%	2	0.20%	3	2.38%	145	1.72%	222	1.97%	145	2.86%	12	0.05%
Firmicutes	4	0.19%	0	0.00%	0	0.00%	1	0.79%	0	0.00%	0	0.00%	1	0.02%	5	0.02%
Gemmatimonadetes	2	0.09%	0	0.00%	0	0.00%	0	0.00%	63	0.75%	100	0.89%	41	0.81%	0	0.00%
Lentisphaerae	3	0.14%	0	0.00%	1	0.10%	0	0.00%	3	0.04%	2	0.02%	1	0.02%	0	0.00%
Planctomycetes	22	1.03%	0	0.00%	0	0.00%	1	0.79%	116	1.37%	87	0.77%	63	1.24%	4	0.02%
SAR406	434	20.24	0	0.00%	30	2.94%	18	14.29	2262	26.79%	2399	21.32%	1035	20.40%	0	0.00%
SAR406_related	20	0.93%	0	0.00%	1	0.10%	0	0.00%	78	0.92%	90	0.80%	18	0.35%	0	0.00%
Verrucomicrobia	17	0.79%	0	0.00%	0	0.00%	2	1.59%	104	1.23%	79	0.70%	63	1.24%	0	0.00%
Others	84	3.92%	0	0.00%	12	1.17%	0	0.00%	195	2.31%	177	1.57%	113	2.23%	14	0.06%
Total	2144		412		1022		126		8444		11250		5073		23999	

Table 4. Composition of pyrosequenced 16S rRNA gene fragments, analyzed in genus or family-level resolution for Alpha- and Gammaproteobacteria, and on phylum- or subphylum-level resolution otherwise. Pyrosequencing fragments were identified in genus- or family-level resolution to identify members of uncultured gamma lineages (for example AGG47, SUP05,

Arctic96BD19, ZD0417, SAR156) or cultured gammaproteobacterial genera of oil degrading heterotrophs (*Oleispira*, *Oleiphilus*, *Marinobacter*, *Alkanivorax*).

1.4 Results and discussion

1.4.1 Bacterial community time lines.

The timeline of bacterial community composition in the aftermath of the Deepwater Horizon blowout reveals a complex pattern of microbial community succession within the oil and gas-impacted water column of the Gulf of Mexico. The baseline for bacterial community composition in the Gulf of Mexico water column on the eve of the Deepwater Horizon blowout is accessible thanks to a serendipitous water sample, collected on March 10, 2010 at 800 m depth at the Mississippi Canyon 118 Microbial Observatory, ca. 9 nautical miles northwest of the Macondo wellhead (Table 1). The 16S rRNA clone library results indicated a water column bacterial community where SAR11 and other *Alphaproteobacteria*, the SAR 406 lineage, the deltaproteobacterial SAR324 lineage, and a complex gammaproteobacterial assemblage of cultured and uncultured lineages, often within the families *Oceanospirillales* and *Alteromonadales*, constituted the dominant proportion (76%) of all clones. Other phyla, such as *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Planctomycetes*, *Verrucomicrobia*, *Gemmatimonadetes* and *Cyanobacteria* were also present (Figure 8). This bacterial community matches, in phylum-level composition and in relative abundance of the major community members, the open-ocean Atlantic and Pacific bacterial communities from the same depth (800 m), as determined by single-cell genome amplification and sequencing (Swan *et al.*, 2011). Thus, the pre-spill deepwater column near the Macondo wellhead shared the microbial community of the ultimate source reservoir of the Gulf of Mexico, the Atlantic Ocean.

In the course of the oil spill, this complex bacterial community was temporarily overprinted by blooms of opportunistic bacteria that responded to the massive influx of

hydrocarbons. The pre-plume bacterial 16S rRNA gene clone library contrasted sharply with the bacterial community composition of the oil-contaminated surface water sample (May 5, 2010) and the hydrocarbon-enriched deepwater plume samples (May 31, 2010). The 16S rRNA gene and pyrosequencing analyses of oil slick-contaminated surface water samples collected shortly after the beginning of the discharge (May 5-9 2010; RV *Pelican*) demonstrated rapid colonization of the surficial oil slick-seawater mixture by PAH-degrading bacteria of the genus *Cycloclasticus*, by oil-degrading members of the genera *Pseudoalteromonas*, *Alteromonas* and *Colwellia*, and by other heterotrophic bacterial groups (Figure 8). This microbial community formed extensive flocs of microbial exopolymeric substances (EPS), observed in the field as microbial flocs developed ubiquitously in the oil-contaminated surface waters in early May 2010 (Passow et al. 2012), and in the laboratory in roller table bottle incubations using fresh oil slick samples and Gulf of Mexico surface water (Ziervogel et al. 2012). Sinking flux of these oil slick-derived microbial EPS flocs exported the associated microbial communities into the deep Gulf of Mexico (Passow et al. 2012).

The clone libraries and pyrosequencing datasets from deep hydrocarbon plume samples (1170 and 1210 m depth) collected on May 31, 2010, were strongly dominated by members of the DWH *Oceanospirillales* cluster; *Cycloclasticus* and *Colwellia* were detected as the most substantial minority population in the pyrosequencing datasets (Figure 8; Table 4). The pyrosequencing datasets detected many bacterial groups in the plume layer that were not visible in the clone libraries, such as Deltaproteobacteria and the SAR406 lineage. The *Oceanospirillales*-dominated enrichment within the plume layer contrasted with the bacterial communities above and below the deep hydrocarbon plume (800 and 1320 m) that resembled the pre- and post-plume clone libraries by the presence – in variable proportions - of SAR11 and

other *Alphaproteobacteria*, *Gammaproteobacteria*, and SAR406; these samples above and below the plume also showed unusually high clone library representation of *Actinobacteria* (14% and 11%), *Planctomycetes* (8% in both depths), and uncultured *Deltaproteobacteria* (5% and 13%). Similar bacterial groups were recovered by pyrosequencing (Figure 8).

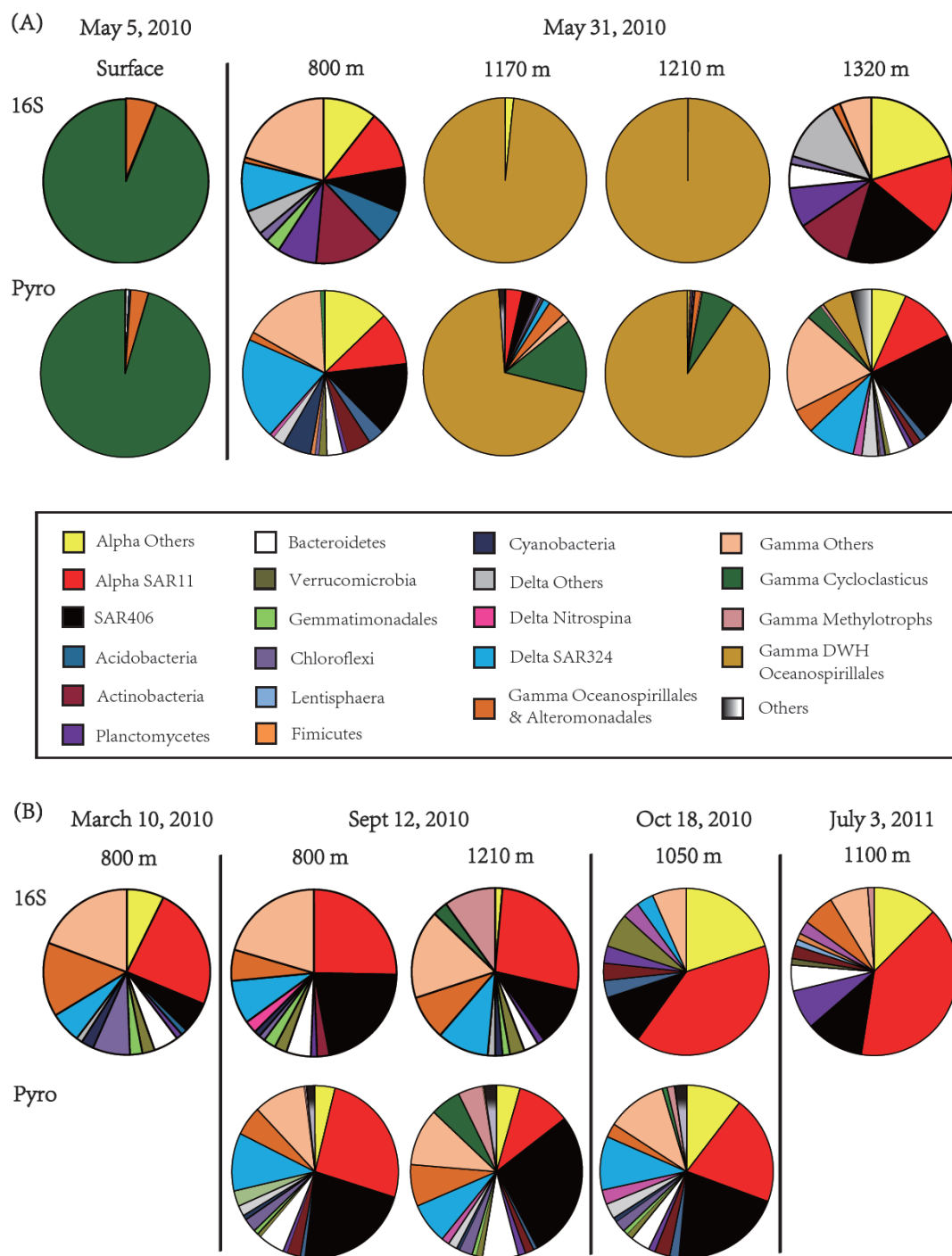


Figure 8. Pie charts of phylum- and subphylum composition of bacterial 16S rRNA gene clone libraries and bacterial 300-bp pyrosequencing fragments from the Gulf of Mexico water column near the Macondo wellhead. The genus *Cycloclasticus* and the DWH *Oceanospirillales* are highlighted. A) Surface water sample collected on May 5, 2010; hydrocarbon plume water column samples near Macondo wellhead from 800, 1170, 1210, and 1320 m depth collected on

May 31, 2010. B) Pre-plume March 2010 sample from 800 m depth near MC118; water column samples from 800 and 1210 m depth near Macondo wellhead, collected September 12, 2010; water column samples from October 18, 2010, and July 3, 2011.

Based on sampling time and location, these water column samples are congruent with previous sampling surveys and bacterial community analyses of the well-documented deep hydrocarbon plume near the Macondo wellhead. Hazen et al. (2010) reported that uncultured members of the gammaproteobacterial order *Oceanospirillales* dominated 16S rRNA gene clone libraries in the deepwater plume between 1100 and 1220 m depth at the end of May 2010 (May 25 to June 2). Subsequent single-cell genome sequencing of two *Oceanospirillales* single cells revealed that they possessed genes involved in the degradation of n-alkanes and cycloalkanes (Mason et al. 2012). This genomic potential of the DWH *Oceanospirillales* is also consistent with the physiological capabilities of their close cultured relatives, *Thalassolituus oleivorans* (Yakimov et al., 2004) and *Oleispira antarctica* (Yakimov et al., 2003), which oxidize long-chain n-alkanes aerobically (Figure 9). Alkane oxidation remains to be checked in the cultured relatives *Bermanella marisrubri* (Pinhassi et al., 2009) and *Oceanoserpentilla haliotis* (Schlosser et al., 2008). Previous analyses show that the hydrocarbon plume had a strong enrichment effect on many heterotrophic genera of marine *Gammaproteobacteria*, whose 16S rRNA gene frequency had increased by 100 to 300% within the plume (Hazen et al. 2010); subsequent microarray-based phylochip analysis of DNA from hydrocarbon plume samples showed increased normalized signal intensity for functional genes involved in hydrocarbon degradation, especially alkane-1 monooxygenase among the alkane and cycloalkane-degrading genes, and a wide spectrum of dehydrogenases, dioxygenases and decarboxylases involved in aromatic carboxylic acid degradation (Hazen et al. 2010; Lu et al. 2012). Most likely, source populations for these genes include cultured heterotrophs and hydrocarbon-degrading bacteria that were

found in our 16S rRNA gene surveys either in plume or post-plume samples, such as *Marinobacter*, *Alteromonas*, *Oleispira*, *Oceanobacter*, *Cycloclasticus*, and uncultured sister lineages of the genera *Saccharophagus*, *Congregibacter* and *Fangia*.

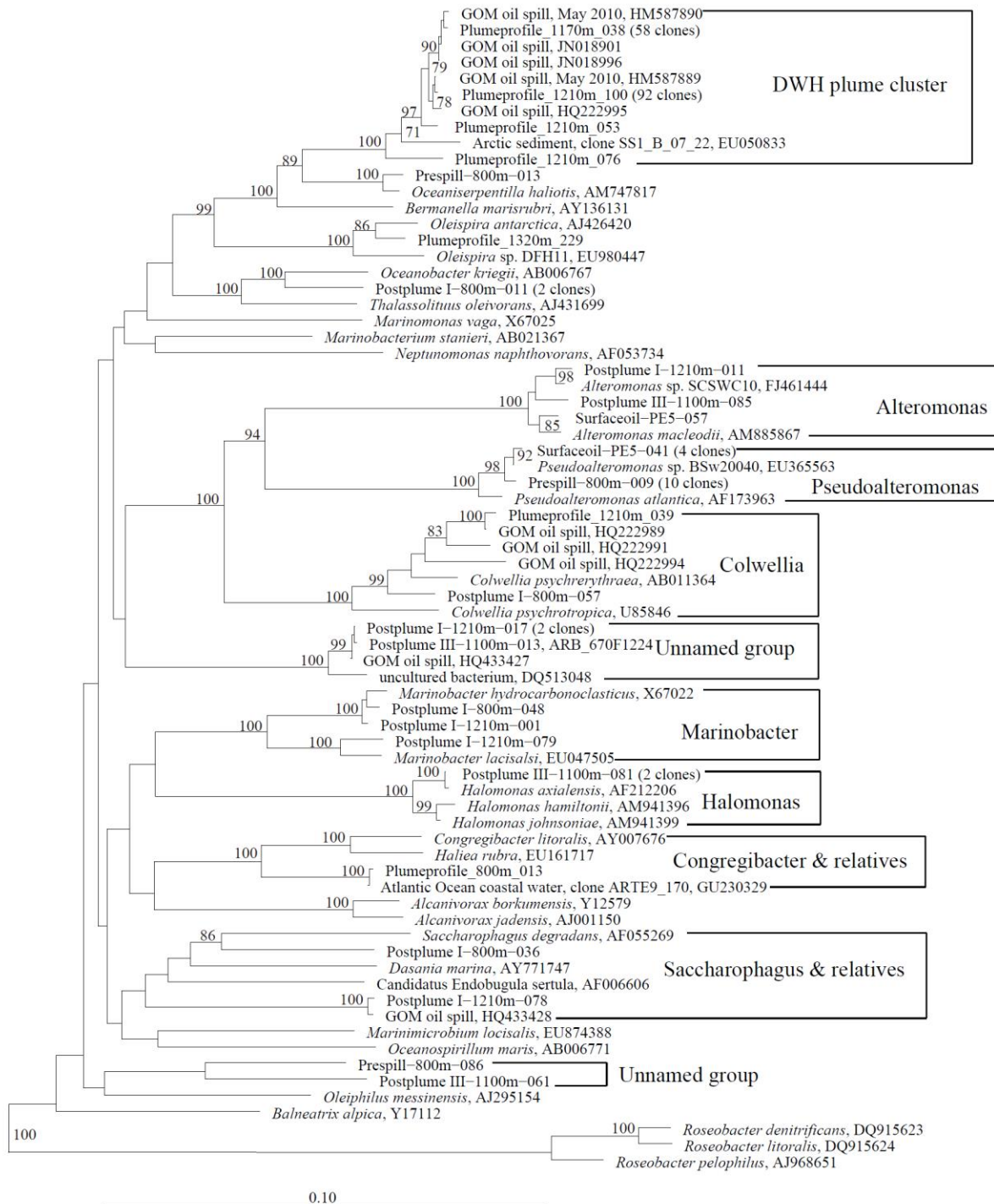


Figure 9. Phylogeny of *Gammaproteobacteria* (*Oceanospirillales* and *Alteromonadales* including DWH *Oceanospirillales*) in the Gulf of Mexico water column near the Macondo wellhead, based on near-full length 16S rRNA genes. Clones from the pre-spill water column sample (March 10, 2010) are labeled “Prespill”; clones from surface oil slicks (May 5, 2010) are labeled “Surfaceoil”; clones from plume water column samples (May 31, 2010) are labeled “Plumeprofile”. Clones from September 12 and October 18, 2010, and from July 3, 2011, are labeled Postplume I, II and III, respectively. The clone designations are followed by sampling depth in meters, and a 3-digit clone ID (Table 2).

The detection of *Cycloclasticus* and *Colwellia* spp. in our pyrosequencing surveys of the plume samples (May 31, 2010) is consistent with the previously published clone library detection of these genera in plume samples from May 26 to June 5 (Redmond and Valentine 2012), and shows that these two oil-degrading genera co-occurred with DWH *Oceanospirillales* in the deep plume (Figure 8). In plume samples collected two weeks later (June 13 to 16, 2010), 16S rRNA gene phylotypes of the genera *Cycloclasticus* and *Colwellia* predominated (Redmond and Valentine 2012); these genera were discussed as bacterial catalysts of the dominant oxygen-consuming process, ethane and propane oxidation, in the deep-water plume (Valentine et al. 2010). This interpretation contrasts with the known substrate spectrum of *Cycloclasticus*, a genus described originally as aerobic degraders of polycyclic aromatic hydrocarbons (Dyksterhouse et al. 1995). *Cycloclasticus* remains recognized as an obligate degrader of these compounds (Yakimov et al. 2005); several *Cycloclasticus* strains were previously isolated from Gulf of Mexico sediments by enrichment with PAH substrates (Geiselbrecht et al. 1998). Thus, a likely role for *Cycloclasticus* is the degradation of BTEX compounds in the plume. The moderately psychrophilic genus *Colwellia*, consistently present in plume- and post-plume samples (Figure 9), was selectively enriched on crude oil at 4 °C (Redmond and Valentine 2012) and was capable of oil degradation at in-situ temperatures of 5 °C (Bælum et al. 2012), consistent with the in-situ temperature of the deep Gulf of Mexico water column. Viewed in context, the bacterial community in the deep plume apparently changed within two weeks from being dominated by

DWH *Oceanospirillales* in late May to becoming dominated by *Colwellia* and *Cycloclasticus* in mid-June (Valentine et al. 2010, Redmond and Valentine 2012).

By mid-September 2010, oxygen depletion signals, CDOM fluorescence and DOSS concentrations showed that the slowly decaying deep hydrocarbon plume drifted in a generally west-southwesterly direction away from the Macondo wellhead area (Kessler et al. 2011; Kujawinski et al. 2011); this is consistent with our CTD profile of the water column near the Macondo wellhead, recorded on Sept 12 2010, that lacks hydrocarbon plume signatures. The post-plume 16S rRNA gene clone libraries and pyrosequencing surveys of September and October 2010, and the 16S rRNA gene clone library of July 2011 shared dominant bacterial groups with the clone library of March 2010, indicating a partial recovery towards the pre-spill bacterial community. The SAR11 *Alphaproteobacteria*, the SAR406 lineage, the deltaproteobacterial lineage SAR324, and a complex assemblage of *Gammaproteobacteria* dominated the clone libraries and accounted together for 81 to 88% of all post-plume clones. The *Planctomycetes*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Chloroflexi* and *Gemmatimonadetes* accounted for smaller proportions or remained undetected in some samples (Figure 8).

Not only dominant phylum-level lineages but also specific pelagic alpha- and gammaproteobacterial lineages reappeared in post-spill clone libraries: the Arctic96BD-19 group of sulfur-oxidizing heterotrophs (Marshall & Morris, 2013) that is prevalent in stratified, oxygen-depleted conditions (Walsh et al. 2009); the SAR11 subclusters (Field *et al.*, 1997); (Figure 11); the uncultured AGG47 cluster associated with marine snow (DeLong et al. 1993); the uncultured North Sea ZD0417 cluster (Stevens & Ulloa, 2008), and the uncultured SAR156 lineage (Mullins *et al.*, 1995) (Figure 10). The widely distributed SUP05 lineage, a presumable sulfur

oxidizer typical of oxygen-depleted water columns (Walsh *et al.*, 2009, Canfield *et al.*, 2010), was found during and after the plume stage.

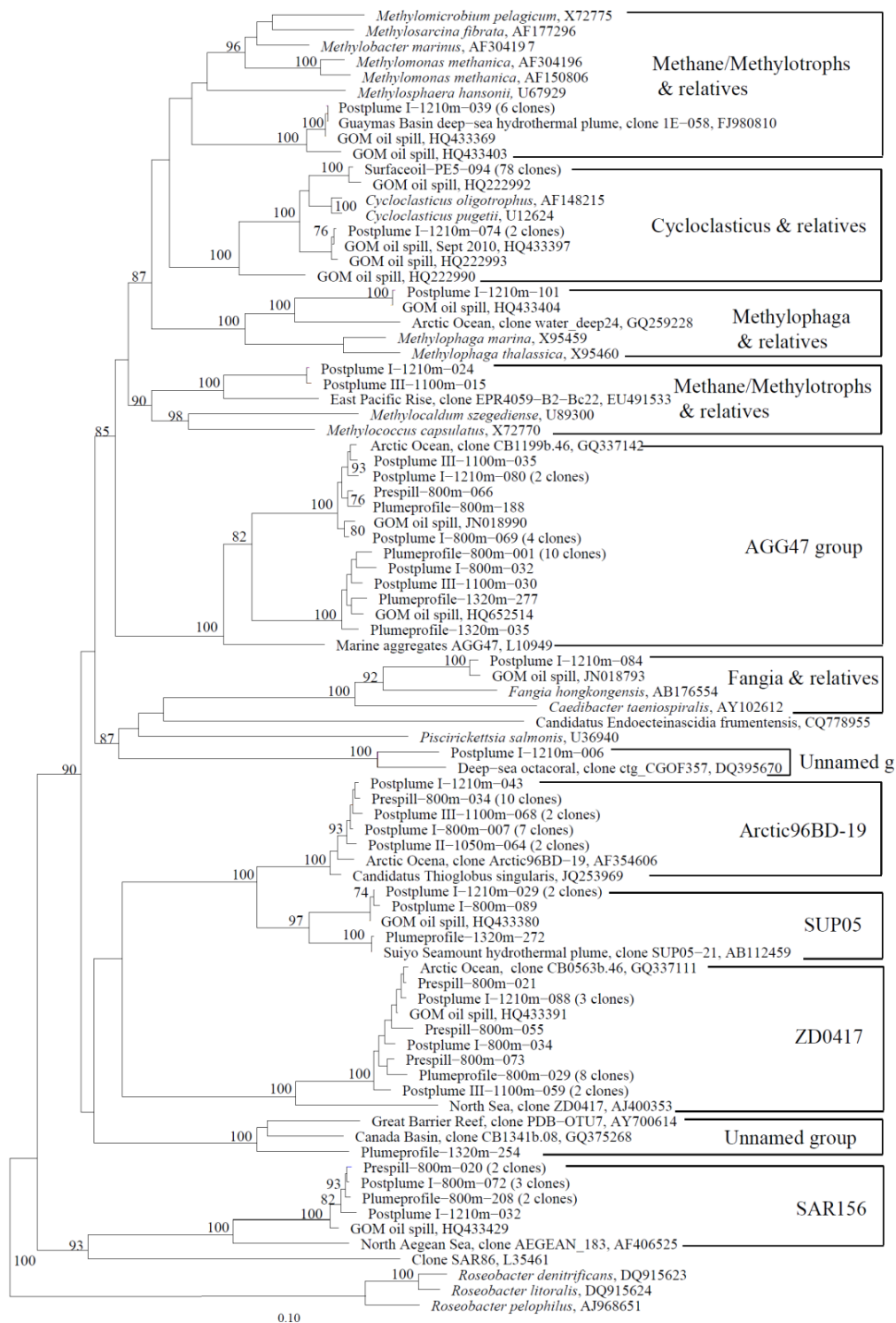


Figure 10. Phylogeny of *Gammaproteobacteria* (Uncultured lineages, *Cycloclasticus* and methaneotrophs/methylotrophs) in the Gulf of Mexico water column near the Macondo wellhead, based on near-full length 16S rRNA genes. Clones from the pre-spill water column sample (March 10, 2010) are labeled “Prespill”; clones from surface oil slicks (May 5, 2010) are labeled “Surfaceoil”; clones from plume water column samples (May 31, 2010) are labeled “Plumeprofile”. Clones from September 12 and October 18, 2010, and from July 3, 2011, are labeled Postplume I, II and III, respectively. The clone designations are followed by sampling depth in meters, and a 3-digit clone ID (Table 2).



Figure 11. Distance phylogeny of SAR11 *Alphaproteobacteria* in the Gulf of Mexico water column, based on near-full length 16S rRNA genes. The subclusters SAR193, SAR203, SAE220, SAR241, SAR407, SAR464 were based on their published phylogenies (Field et al. 1997); the Ingenious Newfangled Group (ING) was defined here. 16S rRNA genes from the pre-spill water column sample (March 10, 2010) are labeled “prespill”; plume water column samples (May 31, 2010) are labeled “Plumeprofile” followed by depth in meters. Post-spill samples (Sept 12, 2010; October 18, 2010, July 3, 2011) are labeled Postplume I, II and III, respectively. Each clone is identified by a 3-digit ID number; see Table 2 for corresponding Genbank numbers.

1.4.2. Pyrosequencing results for surface oil slick and plume-impacted water column.

The pyrosequencing results for the weathered oil mixture at the surface from May 5, 2010, and the water column samples of May 31, 2010 were broadly consistent with the 16S rRNA gene clone libraries for the same samples (Figure 8), but in addition revealed bacterial populations that had remained undetected in the clone libraries (Table 4). In the surface sample, pyrosequencing representation for *Cycloclasticus* (>93%), *Alteromonas* (1.45%) and *Pseudoalteromonas* (1.2%) resembled the clone library results, whereas *Colwellia* and *Halomonas* were detected in smaller proportions. In contrast, the alkane-degrading DWH *Oceanospirillales* accounted for near 90 and 70% of the pyrosequencing reads in the two deep plume samples of late May 2010 (Table 4).

The DWH *Oceanospirillales* pyrosequencing reads were congruent with full-length 16S rRNA gene clones of DWH *Oceanospirillales* from the Gulf of Mexico (Redmond and Valentine 2012) and from the Atlantic Ocean offshore North Carolina (D’Ambrosio 2011), and formed at least three distinct phylogenetic clusters (Figure 12). The pyrosequencing survey also validated a diverse community of hydrocarbon-degrading bacteria in the plume profile that went largely undetected in the clone libraries (Table 4): The PAH-degrading genus *Cycloclasticus* remained variably detectable throughout the water column. Psychrophilic heterotrophs of the genus *Colwellia* (the only group detected in the plume clone libraries besides the DWH *Oceanospirillales*) accounted for approx. 1 to 3 % of the pyrosequencing reads within the

plume. The alkane-degrading genera *Oleiphilus* and *Oleispira* were found in low abundances below and within the plume. The pyrosequencing representation of the uncultured gammaproteobacterial groups (AGG47, Arctic96BD19, SUP05, ZD0417, SAR156) above and below the plume was strongly reduced within the plume (Table 4). A similar trend was observed for *Alphaproteobacteria*. While SAR11 bacteria accounted for a tenth of the pyrosequencing fragments above and below the plume, their representation decreased within the plume (Figure 8). In general, pyrosequencing analysis indicated a functionally and phylogenetically diversified alpha- and gammaproteobacterial community in the hydrocarbon plume; pre-spill populations of uncultured bacteria and oil-degrading bacteria remained detectable against the dominant plume populations of DWH *Oceanospirillales*. This result is compatible with a complex functional gene repertoire of plume microbial communities sampled at the same time (Lu et al. 2012).

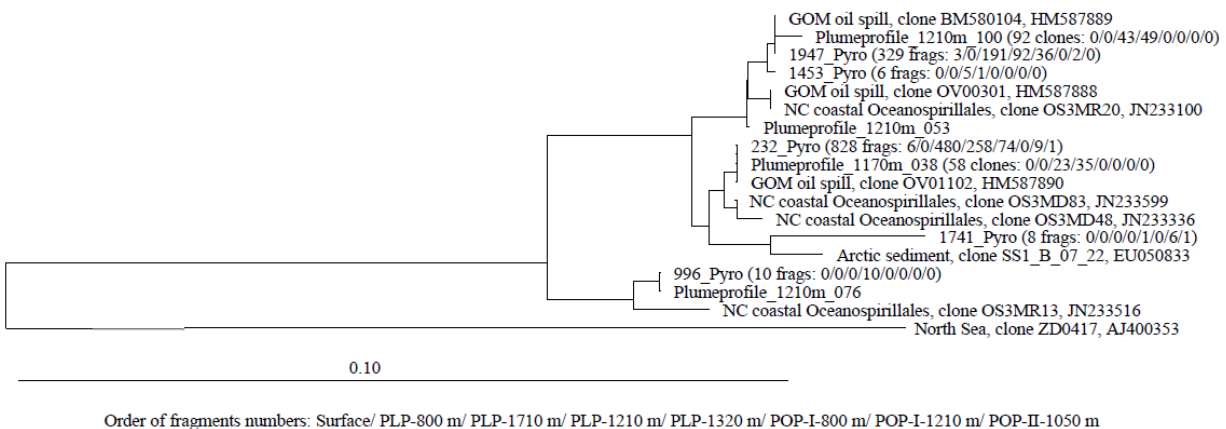


Figure 12. Phylogeny of DWH *Oceanospirillales* based on 300 bp pyrosequencing fragments and corresponding sections of 16S rRNA gene clones, showing the phylogenetic fine structure of this cluster. The phylogeny was obtained with a mask excluded all sequence regions except *E.coli* 16S rRNA gene positions 28-337, equivalent to the pyrosequencing fragment. The number of occurrence for each type of pyrosequencing fragment and 16S rRNA gene clone in the different samples is listed in brackets in the following order: Surface sample; Plume profile at 800 m; Plume profile at 1170 m; Plume profile at 1210 m; Plume profile at 1320 m; Postplume I at 800 m; Postplume I at 1210 m; Postplume-II at 1050 m.

1.4.3. Pyrosequencing results for post-plume water column.

The pyrosequencing results for the post-plume water column samples of September 12, 2010, and October 18, 2010, were broadly consistent with the corresponding 16S rRNA gene clone libraries (Figure 8), but revealed additional bacterial populations that had not been observed in the clone libraries (Table 4). The DWH *Oceanospirillales* that had disappeared from the clone libraries remained detectable at low levels in the pyrosequencing dataset (up to 0.2% at 1200 m, Sept. 12 sample). Interestingly, the post-plume pyrosequencing datasets showed that oil-degrading bacteria persisted in the water column near the Macondo wellhead, although the deep hydrocarbon plume had been drifting in a southwesterly direction, and was no longer detectable in the wellhead region as indicated by CTD profiling in September 2010. Bacterial alkane degraders (*Alcanivorax*, *Oleiphilus*, *Marinobacter*) remained detectable in low proportions (<1%), and the PAH oxidizer *Cycloclasticus* and relatives of gammaproteobacterial methylotrophs accounted for near 5 % of pyrosequencing reads in the 1210 m sample (Table 4). These results suggest local sources that re-inject reservoir populations of these bacteria into the water column, either from small-scale accidental leakage or natural hydrocarbon seepage (Joye et al. 2011b).

Most pyrosequencing fragments from the post-plume water column do not represent specialized oil degraders; these pyrosequencing results resemble (and extend) the diversified 16S rRNA gene clone library results for the same samples. Within the *Gammaproteobacteria*, the cultured genera *Oceanobacterium*, *Oceanobacter*, *Oceanospirillum*, *Alteromonas*, *Pseudoalteromonas*, *Halomonas*, *Idiomarina*, *Marinimicrobium*, *Congregibacter*, were complemented by uncultured water column lineages (two different AGG47 clusters; Delong et al. 1993; Arctic96BD19 and SUP05, Walsh et al. 2009; SAR156, Mullins et al. 1995; a ZD0417-

related group, Stevens and Ulloa 2008). Within the *Alphaproteobacteria*, relatives of the genera *Oceanibaculum* and *Roseobacter*, of the *Rhizobiales*, *Rhodoplanes*, *Rhodospirillales*, *Sphingomonadales*, several uncultured clusters, and the SAR11 lineage (the latter in the 10 to 25% range) were found in all post-plume samples (Table 4). The *Deltaproteobacteria* (dominated by SAR324) and the SAR406 lineage accounted for ca. 10 to 25% of the pyrosequencing dataset, similar to their representation in the 16S rRNA clone libraries (Figure 8). A wide range of phylum-level lineages, the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Gemmatimonadetes*, *Planctomycetes*, and *Verrucomicrobia* accounted for approx. 0.5% to 5% of the pyrosequencing reads (Table 4), and appeared to a limited extent in the corresponding clone libraries (Figure 8). Other phylum-level lineages (Candidate Division OD1, *Epsilonproteobacteria*, *Lentisphaerae*) were barely detected in the pyrosequencing dataset, and were not observed in the clone libraries (Table 4).

1.4.4. Contrasting interpretations of the post-plume bacterial community.

The interpretation of bacterial communities in the water column of late summer 2010 remains contested. Bacterial 16S rRNA gene clone libraries from post-plume water column samples (Sept. 7 to 17, 2010) detected diverse *Alpha*- and *Gammaproteobacteria*, *Flavobacteria*, *Chloroflexi*, and *Planctomycetales* (Kessler et al. 2011); the *Gammaproteobacteria* included *Cycloclasticus*, members of the *Oceanospirillales* (not the DWH group), and members of the *Methylophilaceae*, *Methylococcaceae* and the genus *Methylophaga*. The latter three lineages constitute a phylogenetic assemblage of C₁-oxidizing marine bacteria; this assemblage was regarded as evidence for bacterial methane oxidation as the dominant hydrocarbon-degrading process in the water column during the decay of the deep plume (Kessler et al. 2011), although re-examination of the clone libraries and comparison with substrate spectra of cultured C₁-

oxidizing bacteria suggested that methylotrophy was at least as likely (Joye et al., 2011b). The phylogenetic analysis of these clones and their closest matches reported here shows that they are not representatives of cultured methylotrophic and methanotrophic genera. Instead, they form two separate sister lineages to the methylo- and methanotrophic genera *Methylobacterium*, *Methylosarcina*, *Methylobacter*, *Methylomonas*, and *Methylosphaera*, and to the separately branching, obligately methylotrophic genus *Methylophaga* (Figure 10). If these uncultured bacteria represent methylotrophs or methanotrophs, they would constitute new genera with potentially novel physiological properties. Assuming that these uncultured lineages represent C₁-oxidizing bacteria, the sampling campaign appears to have caught the last stages of a methanotrophic bacterial bloom that pushed the methane concentrations to below typical Gulf of Mexico ambient levels at the time of sampling in September 2010 (Kessler et al. 2011). However, alternative interpretations are possible. Transcriptomics studies that explored the impact of high molecular weight dissolved organic matter on microbial community structure and activity showed a selective enrichment of marine heterotrophs within the *Gamma*- and *Alphaproteobacteria* (*Alteromonas*, *Thalassobius*) and gammaproteobacterial methylotrophs (*Methylophaga*) after a short incubation time (27h) under DOM-amendment (McCarren et al. 2010). These strains could be enriched in consequence of a DOM-degrading heterotrophic cascade that releases naturally abundant methylated sugars from DOM, and leads to the frequently observed high abundance of methylotrophic bacteria in clone libraries from DOM-rich coastal waters (McCarren et al. 2010). In this interpretation, the combined presence of DOM-degrading methylotrophic and heterotrophic *Gammaproteobacteria* and *Alphaproteobacteria* marks the microbial degradation of a DOM pulse; this explanation is consistent with dissolved oxygen and fluorescence anomalies and the lack of detectable methane

at the sampling stations that yielded this bacterial signature (Kessler et al. 2011). The methylotroph-related clones disappeared from the October 2010 clone library, but reappeared in July 2011 (Figures 1, 3). Methylotroph-related sequences remained detectable among the pyrosequencing reads in September and October 2010 (Table 4). Their continued occurrence near the Macondo wellhead and in other widely dispersed marine habitats (for a high-arctic example see Teske et al. 2011) may not be specifically linked to methanotrophy or methylotrophy sustained by fossil hydrocarbons; seasonal phytoplankton blooms provide an alternative explanation that requires systematic investigation.

1.4.5. Natural Reservoirs of DWH *Oceanospirillales*.

The rapid enrichment of specific bacterial types associated with the deep hydrocarbon plume indicates the existence of easily accessible natural reservoirs or seed populations of these bacteria in the Gulf of Mexico. Identifying their natural reservoir is of particular interest toward a more complete understanding of their ecology and adaptability to a massive and prolonged input of oil. The DWH *Oceanospirillales*, for example, lacked closely related representatives in Genbank when first reported (Hazen et al., 2010). The closest relatives in GenBank (EU050833) were a clone from Arctic marine sediments (Tian *et al.*, 2009) and cultured sister groups within the *Gammaproteobacteria*, including the hydrocarbon degraders *Oleispira* and *Thalassolituus*, and the genera *Bermanella*, *Spongispira* and *Oceanoserpentilla* (Hazen et al. 2010). While our pre- and post-plume 16S rRNA gene clone libraries did not contain any full-length DWH *Oceanospirillales* clones, the DWH *Oceanospirillales* were detected by pyrosequencing in the post-plume samples (September and October 2010), indicating a low-level background population and reservoir of these bacteria in the Gulf of Mexico water column.

Unexpectedly, members of the DWH *Oceanospirillales* were found in bacterial 16S rRNA gene and rRNA transcript libraries from the Atlantic shelf break offshore North Carolina, sampled on December 4th, 2009 (D'Ambrosio, 2011), at a depth of 146 m in a distinct water mass known as the Subtropical Underwater (SUW) layer and distinguished by high salinity and warm temperature (Cleroux *et al.*, 2009). They constituted a substantial proportion (around 20% to 25%) of all clone libraries from the SUW sample, regardless of whether these were derived from 16S rRNA genes or 16S rRNA transcripts of the particle-associated or free-living fraction (D'Ambrosio, 2011). The North Carolina *Oceanospirillales* 16S rRNA genes fell into the same phylogenetic clusters as the *Oceanospirillales* 16S rRNA genes and pyrosequencing fragments from the DWH oil spill (Figure 12). Since the North Carolina *Oceanospirillales* were sampled in December 2009, they do not originate from the DWH oil spill; yet they are members of the DWH *Oceanospirillales* cluster by phylogenetic affiliation. The conspicuous enrichment of DWH *Oceanospirillales* in the Subtropical Underwater layer might be the consequence of natural hydrocarbon seepage and hydrocarbon enrichment in this water layer in the southwest North Atlantic (Harvey *et al.*, 1979, Requejo & Boehm, 1985). This North Atlantic population of DWH *Oceanospirillales* could be in constant exchange with the Gulf of Mexico, and might represent a seed population. More generally, the North Atlantic and the Gulf of Mexico occurrences of this microbial group could be the result of local enrichments from a common and widely distributed low-abundance seed population.

1.5 Conclusions

Pyrosequencing and clone library analyses of PCR-amplified 16S rRNA genes and gene fragments have revealed strong microbial community stratification in the deep-plume water column, dominated by abundant populations of alkane-oxidizing DWH *Oceanospirillales* and aromatics-degrading *Cycloclasticus* spp. After the Macondo wellhead was capped and the source

for the deep plume extinguished, the pre-spill pelagic microbial community re-established itself near the vicinity of the Macondo wellhead. However, even after the deep hydrocarbon plume was no longer detectable in the wellhead area in September and October 2010, small populations of oil-degrading *Gammaproteobacteria* and of the DWH *Oceanospirillales* remained detectable by pyrosequencing, indicating persistent and widely occurring seed populations in the water column that respond quickly to natural or anthropogenic hydrocarbon pulses.

CHAPTER 2: OILY MARINE SNOW ASSOCIATED MICROBES²

2.1 Abstract

Oily marine snow aggregates similar to the enormous oily particles formed *in situ* during the spill were generated by using the oil slick collected at the sea surface near the wellhead under lab incubation condition. The composition of these aggregates changed continuously during their formation and precipitation to the seafloor by microbes dwell within the particles. Bacterial community associated with these oily aggregates were mainly composed by *Alphaproteobacteria* (*Roseobacter* clade) and *Gammaproteobacteria* (*Cycloclasticus*, *Halomonas* and *Marinobacter*), together with minority groups *Bacteroidetes*, *Deltaproteobacteria* and *Planctomycetes*. EPS producer *Halomonas* potentially glued the marine snow particles and facilitated the biodegradation process taken by oil-degrading bacteria *Cycloclasticus* and *Marinobacter*. Relatively high abundance of organic matter associated with the aggregates presumably accelerated the growth of heterotrophic bacteria such as *Roseobacter*. The lab incubated oily aggregates shared most of the phylotypes with those from the surface oil slick and sea water bacterial communities, indicating inheriting and continuous development of microbes together with their habitat. Precipitation of these bio-weathered oily snow aggregates with wrapped crude oil and bacteria will affect the microbial community in the seafloor sediment.

² Results of this chapter were published in two articles, the original citation is as follows: Arnosti C, Ziervogel K, Yang T & Teske A (2014) Oil-derived marine aggregates - hot spots of polysaccharide degradation by specialized bacterial communities. *Deep-Sea Research Part II Topical Studies* **Accepted**. Gutierrez T, Berry D, Yang T, Mishamandani S, McKay L, Teske A & Aitken MD (2013) Role of Bacterial Exopolysaccharides (EPS) in the Fate of the Oil Released during the Deepwater Horizon Oil Spill. *PLoS one* 8.

2.2 Introduction

During the oil spill, the estimated area of surface slick (plus oil sheen) was about 1759 square miles (MacDonald, 2010). The amount of oil on the sea surface on May 17 was between 129000 and 246000 barrels (Clark, 2010). Subsurface chemical data show that ~31% of the leaking gas and oil was initially transported in the form of oil droplets up to surface of the ocean and the overlying atmosphere (Ryerson *et al.*, 2011, Ryerson *et al.*, 2012). During the Deepwater Horizon oil spill, a large amount of oil marine snow was observed at the sea surface (Figure 13) (Passow *et al.*, 2012). The floating crude oil gradually changes its composition by losing most of the gas components into air, emulsified to “chocolate mousse”, and suffering from physical and biological weathering. Some of the oil-degrading bacteria, such as *Halomonas*, *Alteromonas*, *Colwellia* and *Pseudoalteromonas* as well as eukaryotic phytoplankton can produce EPS (extracellular polymeric substance) or exopolysaccharides. EPS are high molecular weight polymers composed mainly by monosaccharides (Gutierrez *et al.*, 2013), most polysaccharides are used in industry mainly because of their thickening and gelling properties (Bozzi *et al.*, 1996). The sticky EPS can glue phytoplankton, bacteria, fecal pellet and various organic to form marine snow particles. During this process, marine snow particles keep growing until it loses the buoyance and sinks, therefore, credit a significant contribution to the “biological pump” which transports fixed carbon to deep sea (Alldredge, 2000). Consequently, it is essential to study the microbes dwelling in the oil marine snow flocs for a better understanding of the possible biodegradation within these “hot spots” particles as well as to estimate the oil fallout after the spill at the seafloor.



Figure 13. Mucus-rich marine snow observed in situ beginning of May 2010 in the GoM in the vicinity of oil layers at the surface. Scale bars are 10 cm. (Passow et al., 2012)

2.3 Materials and Methods

2.3.1 Field samples and pure cultures

Field samples. During the RV Pelican's oil spill cruise on May 5, 2010, sample PE5 of mixed surface water and weather oil was collected ca. 0.5 nautical miles from the Macondo wellhead. Twenty-six days later, from an oil spill fast response cruise with RV Walton Smith, samples B1, B6 and B11 were retrieved at 1320m, 1170 m and 800 m depth which, respectively represented waters from below, within and above an deep hydrocarbon plume as discussed in Chapter 1. Sample GIP22 was collected more than 2 months after the wellhead was capped, 37 nautical miles south of the wellhead from 1050 m; it is used as a reference of background *in situ* microbial community of the Gulf. Detailed information of each sample is listed in the Table 5.

Sample ID	Date ^a	Depth (m)	Latitude	Longitude	Distance (mi) ^b
During spill:					
PE5 (surface)	May 5	0	28° 44.175 N	88° 22.335 W	0.86
B1 (below plume)	May 31	1320	28° 41.686 N	88° 26.081 W	3.5
B6 (plume)	May 31	1170	28° 41.686 N	88° 26.081 W	3.5
B11 (above plume)	May 31	800	28° 41.686 N	88° 26.081 W	3.5
Post spill (control):					
GIP22 (water column)	Oct. 18	1050	28° 40.503 N	87° 39.250 W	37.0

^a Collection date in 2010.

^b Distance from spill site in miles.

Table 5. Samples collected from the Deepwater Horizon site.

Pure cultures from the PE5 sample. Isolation of PAH-degrading bacteria from the PE5 surface water sample was performed by streaking 5 µl samples onto ONR7a agar plates that were then individually sprayed with phenanthrene, anthracene, pyrene or fluorine (Kiyohara *et al.*, 1982). The plates were stored in closed plastic bags in the dark at room temperature. Colonies forming clearing zones (indicating degradation of the hydrocarbon had occurred) were picked, purified and examined for growth on other PAH compounds as per the method of Kiyohara *et al.* (Kiyohara *et al.*, 1982). Purified isolates were sequenced and stored frozen at -80 °C in 20% (v/v) glycerol. One of the isolates, TGOS-10, is identical to *Halomonas* sp. strain TG39; since the cultivation conditions were well-known, strain TG39 were used in the consequent experiments.

2.3.2 Oil-aggregate generation by roller bottles

The oil aggregates that are the subject of this study were generated in roller table bottle experiments as described by Ziervogel et al. (Ziervogel *et al.*, 2012). Duplicate 1-L Pyrex glass bottles were filled to the 1-L mark with either uncontaminated seawater (SW) or sea water mixed with 12 ml of oil slick (SW+Oil) yielding an approximate oil slick content of 1% (v/v). In addition, one 1-L glass bottle was filled with 0.1 μm filtered and autoclaved seawater (control SW), and one bottle was filled with control seawater mixed with 12 ml of oil slick (control SW+oil). After keeping all bottles at 25 $^{\circ}\text{C}$ in the dark for 60 hours to let microorganisms adjust to the temperature change, the bottles were incubated at 25 $^{\circ}\text{C}$ on a roller table and were rotated at 3.5 rpm for 21 days in the dark. Bottle rotation introduced small-scale turbulence at the headspace-water interface that enabled us to study biogeochemical processes in the water under mildly turbulent conditions. After 21 days of roller table incubation, the oil-derived aggregates were removed from the SW+oil bottles, combined and slurried with 0.1 μm filtered and autoclaved seawater. A subsample of the slurry was used for DNA extraction, 16S rRNA gene PCR amplification, cloning and sequencing.

2.3.3 Molecular analysis on oil-aggregates and pure cultured bacteria strains

DNA extraction. DNA was extracted from filter samples with oil aggregate material as described previously (Teske *et al.*, 2011). Using sterile forceps, one quarter of each frozen filter was placed and crushed into a 2 ml microcentrifuge tube containing sterile 200 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.5), with 0.5 % (wt/vol) sodium dodecyl sulfate and proteinase K (50 $\mu\text{g mL}^{-1}$). Reactions were incubated at room temperature for 30 minutes followed by the addition of 200 μL equilibrated phenol:chloroform:isoamyl alcohol (25:24:1; pH 8) and vortexing (5 min) at room temperature. The aqueous phase was separated from the phenol

phase by centrifugation at 10,000 x g for 5 minutes, and transferred into a new microcentrifuge tube. The organic phase was re-extracted with another 200 μ L volume of sterile TE (pH 7.5), followed by incubation for 5 minutes at room temperature and phase separation by centrifugation for 5 minutes. From the combined aqueous phases, DNA was precipitated by adding 10% (v/v) 5 M sodium chloride and 2.5 volume of 100% ethanol. Samples were then centrifuged at 13,000 x g for 15 minutes at 4 $^{\circ}$ C. The pellets were washed with 70% ethanol and centrifuged for 15 minutes at 10,000 x g at 4 $^{\circ}$ C. The ethanol was decanted and the pellets were air dried before resuspension in 50 μ L of sterile TE. Total genomic DNA from bacterial isolates was recovered using a Wizard genomic DNA purification kit (Promega, Madison, WI), according to the manufacturer's instructions.

PCR amplification and cloning. The bacterial 16S rRNA gene was amplified with Speedstar DNA polymerase (TaKaRa, Shiga, Japan) using the bacterial primers 8f and 1492r and the manufacturer's recommended concentration for buffer, dNTPs and DNA polymerase. Each PCR reaction consisted of 2 μ L DNA extract, 2.5 μ L 10X FBI buffer (TaKaRa, Shiga, Japan), 2.0 μ L dNTP mix, 2.0 μ L 10 μ M solution of primers 8F and 1492R, respectively (both primers from Invitrogen, Carlsbad, CA), and 0.25 μ L SpeedStar polymerase (TaKaRa), and was brought to 25 μ L with sterile H₂O. Amplification was performed in a BioRad iCycler Thermal Cycler (Hercules, CA) as follows: initial denaturation at 95 $^{\circ}$ C for 4 minutes, 25 cycles of 95 $^{\circ}$ C (10 seconds), 55 $^{\circ}$ C (15 seconds) and 72 $^{\circ}$ C (20 seconds), and a final 10 minute extension of 72 $^{\circ}$ C. PCR product aliquots, including positive and negative controls, were SYBR green stained and visualized using a 1.5% agarose gel. The PCR products were purified using the MinElute[®] PCR Purification Kit according to the manufacturer's instructions (Qiagen, Valencia, CA), and cloned into OneShot[®] TOP10 competent Cells (Invitrogen, Carlsbad, CA) using the TOPO TA

Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer's instructions.

Transformed cells were grown on LB/Xgal/Kanamycin plates. Individual white colonies were arbitrarily picked, re-plated and sanger-sequenced at Genewiz Corporation (South Plainfield, NJ) for oil-aggregates clones and at the UNC Genome Analysis Facility for the isolates, using vector primers M13 F and M13 R. The pyrosequencing method has been introduced in Chapter 1 "Material and Methods".

Phylogenetic analysis. Near-complete 16S rRNA gene sequences from oil aggregates were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) and compared to other sequences via the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/>) (Altschul *et al.*, 1990). After construction of a general 16S rRNA alignment using the ARB phylogeny software package (Ludwig *et al.*, 2004) and the SILVA v95 database (Pruesse *et al.*, 2007), sequences of well-characterized pure cultures and described species of oil-degrading bacteria and marine pelagic bacteria were used for phylogenies whenever possible; otherwise, molecular phylotypes with an informative literature history were selected to anchor major phylogenetic branches of uncultured bacteria. Phylogenetic trees were constructed and bootstrap checks (1000 reruns) of the tree topology were performed using ARB's neighbor-joining function with Jukes-Cantor correction. Sequences were deposited at NCBI Genbank with accession numbers KJ475495-KJ475531. The isolates' accession numbers were labeled in the phylogenetic tree; strain GOS-2, GOS-3a and TGOS-10 with accession numbers JQ246430, JQ246431, JQ246432, respectively. Pyrosequencing data analysis followed the methods in Chapter 1, with *Halomonas* group isolated out and formed an individual *Halomonas* tree, see below.

2.4 Results

2.4.1 Hydrocarbon degradation by *Halomonas* sp. strain TG39 and amphiphilic properties of its EPS

Utilization of hydrocarbons. Various substrates were tested for growth or mineralization by the strain TG39 (Experiment performed by Tony Gutierrez) (Gutierrez *et al.*, 2013). Growth was observed on salicylate, catechol, phenol, benzoate, naphthalene and hexadecane. Weak growth was recorded on fluoranthene, pyrene and chrysene, and no growth was measured on decane, benzene, toluene, p-xylene, biphenyl, fluorene, phenanthrene, anthracene and benzo-pyrene. Strain TG39 did not significantly mineralize phenanthrene, anthracene, pyrenen, benzo[a]pyrene, chrysene, decane and hexadecane compared to their respective acidified controls (Gutierrez *et al.*, 2013). The strain's ability to mineralize p-cresol, o-cresol, naphthalene and fluoranthene, and produce weak growth on pyrene, is reflected in its ability to assimilate phthalate, salicylate, catechol and benzoate which are common intermediates in PAH degradation (Gutierrez *et al.*, 2013).

Relative abundance during the spill. Bar-coded 16S rRNA gene pyrosequencing was used to analyze the bacterial communities present in the 5 water samples from the Gulf of Mexico collected during (PE5, B1, B6, B11) and after (GIP22) the spill. A complete presentation of the community structure represented by these libraries is reported in Chapter 1. A total of 23999, 2186, 133, 412 and 6498 high quality partial gene sequences were obtained for the PE5, B1, B6, B11 and GIP22 libraries, respectively, from which five *Halomonas* phlotypes were identified across these libraries and their relative abundances shown in Table 6. All five phlotypes were identified in the PE5 library. Of these, highest sequence similarity ($\geq 97\%$) to isolate GOS-2 occurred with OTU_319 and OTU_2155; to isolate GOS-3a, with OTU_2155,

OTU_2215 and OTU_2254; and to isolate TGOS-10, with OTU_319 and OTU_2215. Only one of these *Halomonas* phylotypes, OTU_2155, was identified in the GIP22 library. These and other related sequences were used to construct the neighbourjoining tree displayed in Figure 14. Our analysis did not reveal any *Halomonas*-related sequences in the B1, B6 and B11 pyrosequencing libraries. The change in abundance of *Halomonas* 16S rRNA gene sequences, and of other bacterial genera recognized for their EPS-producing abilities, in contaminated surface water (PE5 sample) during the spill are shown in Figure 15, expressed relative to their abundance in the uncontaminated reference sample (GIP22). *Halomonas*, *Colwellia*, *Alteromonas* and *Pseudoalteromonas* were enriched by 1550%, 1950%, 3390 and 14250%, respectively. These organisms were not detected in the other water column samples, with the exception of *Colwellia* which was found enriched in plume water (B6 sample) by 1450% relative to the reference sample.

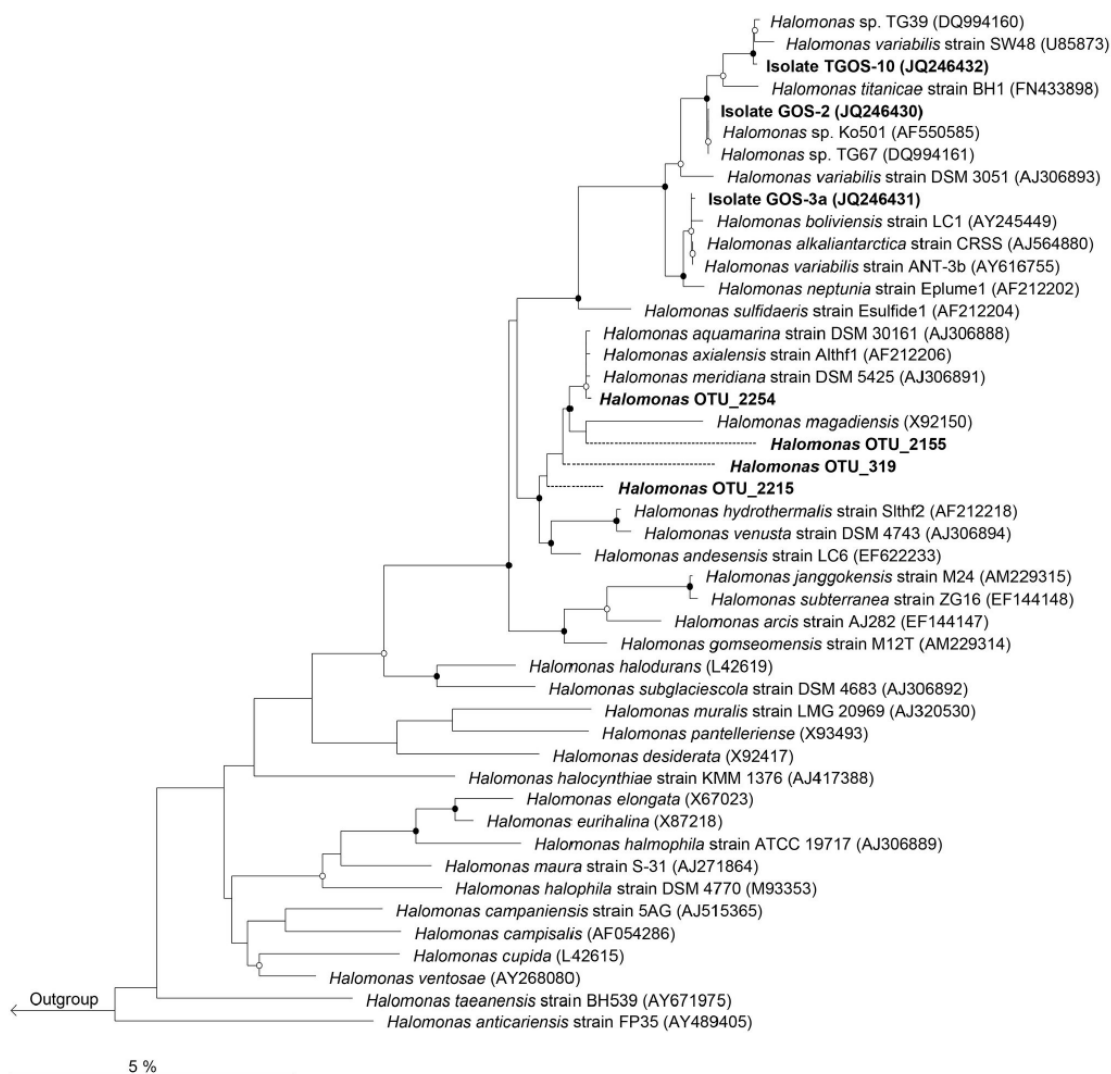


Figure 14. Neighbor-joining phylogenetic tree. The tree, which uses the Jukes-Cantor model of evolution, is based on 16S rRNA gene sequences (>1,200 bp) showing the relationships between isolates GOS-2, GOS-3a, TGOS-10 and representatives of related taxa. These isolates clustered together with several other EPS-producing halomonads that are marked with an asterisk. Filled circles indicate nodes with bootstrap values (1,000 bootstrap replications) greater than 90%; open circles indicate bootstrap values greater than 60%. Pyrosequence phylotypes with >97% sequence identity to any one or more of the *Halomonas* isolates were added using the ARB maximum parsimony quick-add feature (dashed branches). GenBank accession numbers are shown in parentheses. *Zymobacter palmae* (D14555) was used as an outgroup. Bar, 5 substitutions per 100 nucleotide positions. (Gutierrez *et al.*, 2013)

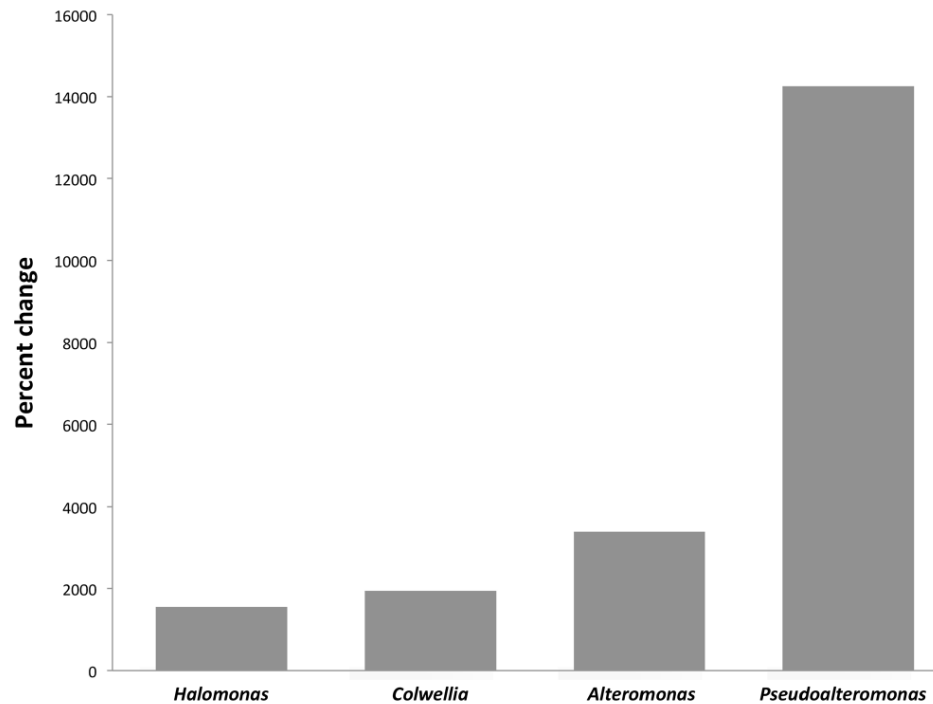


Figure 15. Enrichment of various exopolysaccharide (EPS)-producing bacteria during the Gulf of Mexico oil spill. Percent change in abundance of 16S rRNA gene sequences in contaminated surface water collected during the Deepwater Horizon oil spill. Changes were calculated relative to the post-spill reference sample GIP22. (Gutierrez *et al.*, 2013)

OTU_ID	During spill (May 2010):				After spill (Oct. 2010):	Isolates with >97% 16S rRNA similarity
	PE5	B1	B6	B11	GIP22 (Control)	
319	0.03	0	0	0	0	GOS-2, TGOS-10
801	0.02	0	0	0	0	none ^b
2155	0.01	0	0	0	0.01	GOS-2, GOS-3a
2215	0.03	0	0	0	0	GOS-3a, TGOS-10
2254	0.05	0	0	0	0	GOS-3a
Total (%):	0.14	0.0	0.0	0.0	0.03	

^a Percentage of *Halomonas* sequences of total reads in each library.

^b Low 16S rRNA sequence identity (<95%) to *Halomonas* isolates GOS-2, GOS-3a and TGOS-10.

Table 6. Relative abundance (%) of *Halomonas* phylotypes identified in 16S rRNA gene pyrosequence libraries^a

2.4.2 Formation of oil aggregates in roller bottles

The roller bottles contain uncontaminated seawater collected near the Deepwater Horizon site amended with surface oil sampled in the same area (SW+oil) led to rapid formation of aggregates within one day (Ziervogel *et al.*, 2012). The big aggregates formed after 7 days; they were similar in size (up to 30 mm in diameter) and appearance compared to oil aggregates observed in surface waters near the spill site, with visibly incorporated oil droplets since the formed gelatinous networks was very sticky (Ziervogel *et al.*, 2012) (Figure 16). Aggregate formation in the control samples with only natural seawater was delayed, and reduced in size and abundance. The roller bottle experiment was performed by Kai Ziervogel.

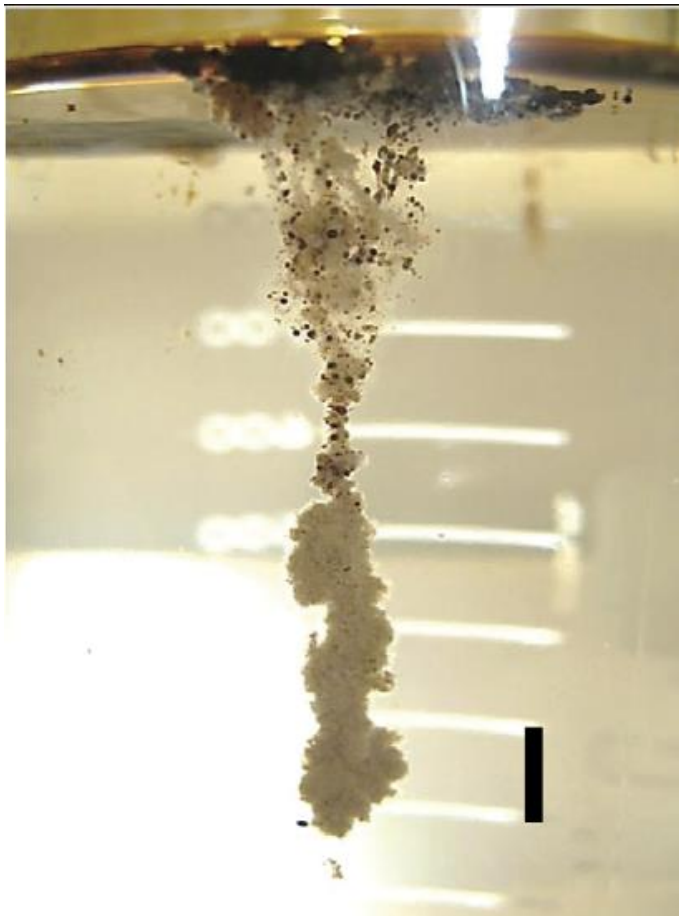


Figure 16. Photo of an oil aggregate formed in one of the roller bottles. Oil aggregate attached to surface water oil slick through sticky oil gels. Photo was taken at the end of the 21-day roller

table incubation in one of the roller bottles containing seawater and oil (SW+oil1). Scale bar is approximately 10 mm. Credit: Kai Ziervogel

2.4.3 Bacterial community composition within the oil aggregates

The bacterial community from aggregates yield by the end of the SW+oil treatment in roller bottle experiment was analyzed. The 16S rRNA gene clones predominantly belong to alpha- and Gammaproteobacteria, with some clones affiliated to the phyla *Bacteroidetes* and *Planctomycetes*, and a *Deltaproteobacteria* group (Figure 17). The gammaproteobacterial clones (a total of 29) were mostly affiliated with the obligately PAH-degrading genus *Cycloclasticus*, with the heterotrophic genera *Congregibacter* (Fuchs *et al.*, 2007) and *Haliela* (Urios *et al.*, 2008, Urios *et al.*, 2009) within the family Alteromondalae, and with the alkane-degrading heterotrophic marine genera *Halomonas* and *Marinobacter* (Figure 17). More than half of the alphaproteobacterial clones (12/22) were affiliated with the metabolically versatile marine *Roseobacter* cluster; rest of them fell into the genus *Hyphomonas* and into several unidentified lineages (Figure 17). Within the phylum *Bacteroidetes*, a total of four clones were affiliated with the heterotrophic, aerobic flavobacterial genera *Krokinobacter* (Khan *et al.*, 2006) and *Coccinimonas*, and the sphingobacterial genus *Fulgivirga* (Nedashkovskaya *et al.*, 2007). The *Planctomycetes* clones (a total of 5) were distantly affiliated to the marine heterotrophs *Phycisphaera mikurensis* (Fukunaga *et al.*, 2009) and - more closely - to *Planctomyces maris*. The sole deltaproteobacterial clone branched with the genera *Kofleria* (Euzéby, 2007) and *Halangium* (Fudou *et al.*, 2002) within the heterotrophic marine *Myxococcaceae* (Figure 17).

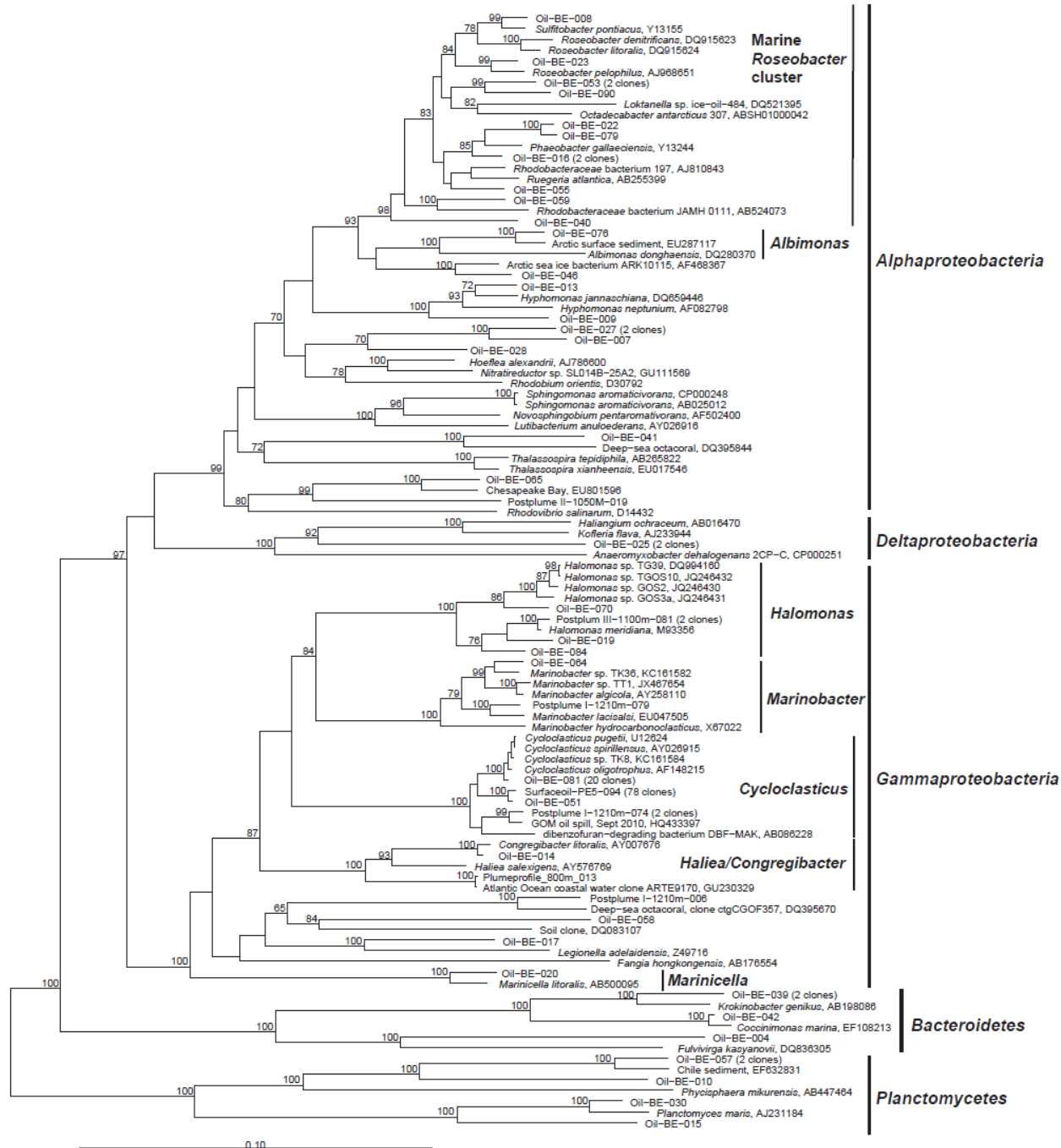


Figure 17. Distance phylogeny of bacterial 16S rRNA genes recovered from the bottle-enriched oil aggregates based on near-complete 16S rRNA gene sequences. Clones labeled “BE” were obtained from the oil aggregates produced in roller table bottle incubations (Ziervogel et al. 2012). Clones labeled “Plumeprofile” and “Postplume” are from water column study as showed in Chapter 1

2.5 Discussion

During the marine snow aggregation, many phytoplankton and bacteria release EPS that enhance the process; one of the bacteria is *Halomonas*, which has been isolated here from the surface oil water (Gutierrez *et al.*, 2013, Gutierrez *et al.*, 2013). Many *Halomonas* species are recognized for producing moderate to large quantities of EPS that can exhibit amphiphilic, or biosurfactant-like, properties. Several reports have shown that these polymers effectively emulsify hydrocarbons, crude oils or refined petroleum products (Bouchotroch *et al.*, 2000, Pepi *et al.*, 2005, Mata *et al.*, 2006, Gutierrez *et al.*, 2009). The EPS produced by *Halomonas* sp. strain TG39 has been investigated its ability to promote the dissolution and biodegradation of hydrocarbons, with particular reference to the role that EPS-producing bacteria contributed to the fate of the oil released from the Deepwater Horizon blowout (Gutierrez *et al.*, 2013). The initial data showed that the strain TG39 is able to degrade and/or mineralize various hydrocarbons, and it secretes polymers that effected the emulsification of oil help the versatility of oil degradation (Gutierrez *et al.*, 2013). Previous work with the EPS of strain TG39 showed that it effectively emulsified n-hexadecane and several food-grade oils (Gutierrez *et al.*, 2007), as well as several aromatic hydrocarbons, phenanthrene, pyrene, fluorene and biphenyl (Gutierrez *et al.*, 2013). The increased solubility of each tested hydrocarbon derived by higher concentration of EPS well supported the essential function of EPS for helping the emulsification. This is especially useful in sea water, there is an inverse relationship exists between salinity and hydrocarbon solubility (Whitehouse, 1984, Gutierrez *et al.*, 2013). Thus, organisms such as strain TG39 may be expected to provide an advantage to other marine bacteria that would otherwise be limited in their capacity to gain access to these hydrocarbon compounds. By adding the strain TG39 EPS into a phenanthrene-amended, natural sea water sample collected at the Deepwater Horizon site, Tony et al. (Gutierrez *et al.*, 2013) found the first line of evidence that implicate *Halomonas* EPS

in enhancing the degradation of oil hydrocarbons in the marine environment by increasing the dissolution and bioavailability of these compounds to indigenous oil-degrading microorganisms, such as during the Deepwater Horizon oil spill.

Though the relative abundance of *Halomonas* in oil contaminated surface waters at Deepwater Horizon was quite low, they were by no means inactive since several *Halomonas* phylotypes were enriched (by 1550% collectively) as a result of the spill compared to the reference sample (GIP22) where all but one of the *Halomonas* phylotypes were undetected. Hazen et al. (Hazen *et al.*, 2010), for instance, also reported the enrichment of *Halomonas* by 140% in the deepwater oil plume compared to the abundance of this group in uncontaminated reference waters. The absence of 16S rRNA fragment reads in the other water column samples collected during the spill (B1, B6, B11) may be explained by the lower sequencing depth (total number of reads) in these pyrosequence libraries compared to that of the surface water PE5 library. In addition to *Halomonas*, other types of EPS-producing bacteria were markedly enriched in surface waters during the spill, which by nature of their potential to produce and release EPS, may similarly have contributed to the fate of the oil. Notably, *Colwellia*, *Alteromonas* and *Pseudoalteromonas* were enriched by at least one order of magnitude compared to their abundance in uncontaminated reference waters. These organisms collectively represented ca. 3% of total bacterial sequences in the PE5 library. Since strains of these genera have been well characterized for their ability to produce EPS (Bozzi *et al.*, 1996, Jouault *et al.*, 2001, Cambon-Bonavita *et al.*, 2002, Nichols *et al.*, 2004, Qin *et al.*, 2007, Marx *et al.*, 2009), their enrichment in surface waters during the spill may have also stimulated their production and release of significant quantities of EPS. Considering the potential of marine bacterial EPS to interface with oil hydrocarbons and enhance the bioavailability of these compounds for microbial

degradation, we posit that these types of bacteria contributed significantly to the fate of the oil released into the Gulf of Mexico via their production and release of EPS.

Supporting this hypothesis, insights on the formation of oil aggregates during the Gulf spill also point to an important role for bacterial EPS in the fate of the oil. Passow et al. (Passow *et al.*, 2012) observed the marine snow formation by using different types of oil contaminated waters (surface oily waters and plume waters from various time points) supplied with the Gulf of Mexico oil or the Louisiana reference crude oil, and came out 3 mechanisms to the snow particle formation. Participation of physical, chemical as well as biological activities are required in the marine snow formation, and existence of stringy mucus web is critical to all of the 3 mechanisms. Therefore, the EPS produced by phytoplankton and bacteria slough off cell surfaces as nanofilbers (Leppard, 1997), which can self-assemble and coalesce to form larger gels and porous networks. These sticky particles enable aggregation by providing the glue and the matrix of phytoplankton aggregates (Passow *et al.*, 1994). Our roller bottle experiment generated big oily marine snow flocs that is very similar to the mucus-rich marine snow observed *in situ* in the vicinity of oil layers at the wellhead area in May 2010 (Figure 16). Results of our roller bottle experiment (Ziervogel *et al.*, 2012) showed that oil aggregates yielded high levels of peptidase and β -glucosidase activities, indicating that a major component of their composition is glycoprotein. This is consistent with the composition of marine bacterial EPS (Nichols *et al.*, 2004, Gutierrez *et al.*, 2007), including that from many *Halomonas* species such as strains TG39 and TG67 (Gutierrez *et al.*, 2007) – strains with 100% 16S rRNA gene sequence identity to, respectively, the strains TGOS-10 and GOS-2 which we isolated from the Gulf spill site. These oil aggregates are hot spots of microbial activity: the patterns of polysaccharide-hydrolyzing enzyme activities within the aggregates were very different from those in the water surrounding

the aggregates after formation, and in surface water that did not contain the oil (Arnosti, *et al.*, 2014, accepted). Specific oil aggregate-associated hydrolysis rates were also more than three orders of magnitude greater than in the water surrounding the aggregates. The differences in initial hydrolysis profiles, and in evolution of these profiles with time, point to specialized metabolic abilities among the oil-aggregate communities compared to oil-water and ambient water communities.

The bacterial community within our roller bottle generated oil aggregate was dominated by *Gammaproteobacteria* and *Alphaproteobacteria*, more than half of which belong to the marine *Roseobacter* clade. Distinguishing features in the oil aggregates analyzed here are the absence of the dominant water column populations such as the SAR11 *Alphaproteobacteria* and SAR324 *Deltaproteobacteria* typically seen in uncontaminated water, and the Deepwater Horizon *Oceanospirillales* found in the deep hydrocarbon plume, and the dominance of the genus *Cycloclasticus* in these aggregate samples. Of the *Cycloclasticus* clones in the oil aggregates, twenty were nearly identical to each other, to described *Cycloclasticus* species *C. pugetii*, (Dyksterhouse *et al.*, 1995); *C. spirillensus*, (Chung & King, 2001); *C. oligotrophus*, (Button *et al.*, 1998), and to *Cycloclasticus* strain TK-8 isolated from oil-contaminated surface water sampled near the DWH site, using naphthalene and phenanthrene as substrates (Gutierrez *et al.*, 2013). The published *Cycloclasticus* species, the new isolate TK-8 from surface oil slicks, and oil aggregate enrichments from the roller table bottle experiment showed near-identical 16S rRNA gene sequences (Figure 17). Interestingly, a slightly different, distinct *Cycloclasticus* phylotype dominated the weathered oil and seawater mixture sampled in early May. This phylotype was recovered repeatedly (78 clones) by sequencing of the weathered surface oil sample PE5 that provided the inoculum for the bottle experiments (Ziervogel *et al.*, 2012), which

in turn generated the aggregates analyzed here. The oil aggregates contained only a single clone of this *Cycloclasticus* phylotype. Therefore, the sequence of experiments – from environmental sample to laboratory-generated oil aggregates - was accompanied by a shift between the two *Cycloclasticus* phylotypes.

The genus *Halomonas* appeared in the oil aggregate with three clones related to it (Figure 17), well support the physiological role of producing EPS for this genus of marine heterotrophs in oil aggregates. It coincides with our *Halomonas* produced EPS results that verified EPS exhibiting amphiphilic properties that allow these macromolecules to interface with hydrophobic substrates, such as hydrocarbons (Bouchotroch *et al.*, 2000, Martinez-Checa *et al.*, 2002, Pepi *et al.*, 2005, Mata *et al.*, 2006, Gutierrez *et al.*, 2007, Gutierrez *et al.*, 2013). At the same time, *Halomonas* species can be enriched from crude oil or oil-contaminated soil and sediment (for example, (Wang *et al.*, 2007) using alkanes (hexadecane) as substrates (Pepi *et al.*, 2005, Mnif *et al.*, 2009). Our also obtained EPS-producing *Halomonas* strains were isolated from DWH surface oil slicks in our isolation experiment. Thus, the conclusion that *Halomonas* spp. catalyze oil degradation in two ways, directly as an oil-degrading bacterium and indirectly as an EPS-producing emulsifying agent is confirmed again by our aggregate 16S rRNA clone library result.

A phylotype within the genus *Marinobacter* represents a third group of oil-degrading bacteria in the oil aggregates (Figure 17). *Marinobacter* species are readily isolated from marine aggregates (Kaeppel *et al.*, 2012), but also from oil wells (Huu *et al.*, 1999). Several species are capable of hydrocarbon (alkane) degradation (Gauthier *et al.*, 1992, Huu *et al.*, 1999), and *Marinobacter* spp. are commonly enriched by marine oil spill contamination (Head *et al.*, 2006, Yakimov *et al.*, 2007). *Marinobacter* strains TK-36 and TT1 were isolated from surface water and from the deep hydrocarbon plume near the DWH site with hexadecane as the sole substrate;

Marinobacter spp. were also enriched in stable isotope probing experiments using deep hydrocarbon plume water as inoculum and ^{13}C -labeled hexadecane as substrate (details in Chapter 4). Thus, *Marinobacter* spp. might have a double function similar to *Halomonas*, as alkane degrader and as EPS-producing emulsifier.

At present, working hypotheses on specific oil-degrading activities and ecological roles for the gammaproteobacterial phylotypes related to the marine genera *Haliella* or *Congregibacter* are lacking. *Congregibacter* is a facultative anaerobic anoxygenic photosynthesizer (AAnP) and an oligotrophic specialist (Fuchs *et al.*, 2007); *Haliella* has been described as a genus of marine heterotrophs isolated from marine coastal waters (Urios *et al.*, 2008, Urios *et al.*, 2009).

In contrast to bacteria that specialize in the degradation and assimilation of petroleum components, most bacterial community members in the oil-aggregates are related to bacterial species and genera that have a broad range of growth substrates and do not depend exclusively on petroleum compounds (Figure 17). They appear to be involved in the secondary degradation of oil-derived biomass and EPS (Head *et al.*, 2006), and could be candidates for production of extracellular enzymes whose activities were measured in these experiments. Phylotypes of the flavobacterial genera *Krokinobacter* and *Coccinimonas* within the phylum *Bacteroidetes*, isolated from coastal marine sediments in Japan (Khan *et al.*, 2006), of the metabolically versatile marine *Roseobacter* clade and other family-level groups within the *Alphaproteobacteria* (Buchan *et al.*, 2005, Lee *et al.*, 2005, Brinkhoff *et al.*, 2008), of the *Myxococcaceae* within the *Deltaproteobacteria*, and of uncultured lineages within the phylum *Planctomycetes*, most likely represent secondary oil-degrading bacterial community members in the oil aggregates.

2.6 Conclusion

Surface oil slick formed large amount of marine snow in a short time, these oil aggregates are responsible for the majority of the downward transport of oil by gravitational settling.

Bacteria that release EPS play an important role in hydrocarbon degradation and marine snow formation, since the produced EPS can increase the solubility and emulsification of hydrocarbons in seawater, as well as coagulate oil droplets and marine debris, thus promote the biodegradation possibility. Oil-degrading bacteria and EPS-producing bacteria found in the roller bottle generated oil aggregates indicate very active biodegradation, especially the genus *Cycloclasticus* and its sister group have been found in both oil slick and the oil aggregates, indicated changing hydrocarbon in the oil marine snow aggregates.

CHAPTER 3: SUCCESSIONAL SEDIMENT BACTERIAL COMMUNITY DYNAMICS IN THE AFTERMATH OF THE DEEPWATER HORIZON OIL SPILL

3.1 Abstract

The huge load of hydrocarbons during the Deepwater Horizon oil spill directly changed the bacterial community structures in the sediment close to the Macondo wellhead. Compared to the 16S rRNA clone libraries from unpolluted sediments in early May 2010, the September 2010 bacterial clone libraries from oil-contaminated sediments shows a sharp increase in *Alphaproteobacteria*, in particular members of the *Roseobacter* cluster, and members of the phylum *Verrucomicrobia*. In October 2010, sediments near the wellhead yielded mostly phylotypes of the phylum *Bacteroidetes*, and the *Delta*- and *Gammaproteobacteria*; the latter two included the sulfate-reducing bacterial families *Desulfobacteraceae* and *Desulfobulbaceae*, and the obligately aromatics-degrading genus *Cycloclasticus* that had previously been found both in surface oil slick and the deep hydrocarbon plume. The *Desulfobacteraceae* and *Desulfobulbaceae* clones did not appear in non-polluted surface sediments, and differed from the sulfate reducers commonly found at natural hydrocarbon seeps in the Gulf of Mexico. The changing bacterial community structure indicated that the oil-derived sedimentation pulse triggered bacterial community perturbations and possibly created patchy anaerobic micro-environments that favored sulfate-reducing bacteria, even at the sediment/seawater interface. The *Planctomycetes* increased from low clone library proportions in October 2010 towards higher representation in November 2010 and in July 2011, one year after the oil spill. Sediment

microbial community dynamics reveals the deposition of the oil-derived sedimentation pulse together with its continuing microbial processing.

3.2 Introduction

The Deepwater Horizon oil spill released more than 4.9 million barrels of crude oil into the Gulf of Mexico (McNutt *et al.*, 2011). The oil budgets showed that in total about 25% of the spilled oil was recovered or skimmed and burned, 23% was evaporated or dissolved, 29% was dispersed chemically (16%) or naturally (13%), with the rest 23% with no clear environmental consequences (McNutt *et al.*, 2011). The surface oil slick rapidly emulsified after the onset of the spill and formed a large amount of oily marine aggregates at the sea surface that subsequently started to sink (Passow *et al.*, 2012). As inferred from laboratory oil incubation experiments and in-situ observations, the floating oil aggregates gradually lost their buoyancy due to EPS-catalyzed aggregation with higher density particles (Arnosti *et al.*, 2014) (Passow *et al.*, 2012), and eventually transported partially weathered oil down to the seafloor and seriously impacted the benthic ecosystem, a process referred to as the “Dirty Blizzard” (Hollander *et al.*, 2013). In late August 2010, the total petroleum hydrocarbons (TPH) concentration of sediment collected near the wellhead had exceeded the EPA standard (Zukunft, 2010). During a response cruise with R/V *Oceanus* (Aug. 21 – Sept 16, 2010), multicores of olive-gray seafloor sediment showed a 2-3 cm thick fluffy red-brownish surface layer sitting on top (Figure 19) which emitted a strong gasoline smell and contained oil aggregates of various sizes, (cruise blog: <http://www.joyerresearchgroup.uga.edu/posts/focusing-oil>). The red-brown color derived from iron- and manganese oxides that precipitated in the upper sediments after being mobilized by oil-stimulated metal reduction (Hastings *et al.*, 2014).

Subsequent metagenomic studies analyzed the microbial community response in oil-impacted sediments during the fall of 2010. From a total 64 sediments surveyed between

September 16 and October 20, 2010, the 19 samples nearest to the wellhead contained high concentrations of total petroleum hydrocarbon (TPH; average 19258 $\mu\text{g kg}^{-1}$) that exceeded the EPA aquatic life benchmarks for polycyclic aromatic hydrocarbons (PAHs) (Mason *et al.*, 2014). Metagenomic analysis indicated that the surface sediment microbial community was structured according to the concentrations of TPH and inorganic nitrogen ($\text{NH}_3 + \text{NO}_3$). Functional genes involved in degrading alkanes and aromatic hydrocarbons increased with hydrocarbon deposition, but functional genes for PAH degradation did not change significantly in abundance compared to non-polluted sediments (Mason *et al.*, 2014). Denitrification genes were recovered preferentially from oil-impacted sites compared to non-polluted sediments; aerobic nitrification was also predicted based on the metagenomic data. These metagenomic indicators suggested oxygen depletion and changing redox conditions in the surficial sediments following the marine snow-derived oil pulse (Mason *et al.*, 2014). Another metagenomic study using three samples just below the sediment surface (1.5-3 cm) from the same cores showed that *Deltaproteobacteria* and anaerobic functional genes were found preferentially in the sample closest to the wellhead (Kimes *et al.*, 2013). In comparison, sediment samples collected 2-6 km from the wellhead one year after the spill contained high abundance of *Methylococcus*, *Methylobacter*, *Actinobacteria*, *Firmicutes* and *Chloroflexi*, provided a snapshot of the microbial community at a later stage of oil spill impact and community succession (Liu & Liu, 2013). Although metagenomic surveys with a deeply sequenced dataset provide a broad view of the possible functions of the surface sediment microbes, the narrow time windows represented by the sediment samples do not reveal the taxonomic shifts within the benthic bacterial community over time. Our time series survey of sediment microbial community dynamics covered the period from May 2010, approx. 2 weeks after the spill occurred, to July 2011, more than one year after the wellhead was capped; the

survey revealed a distinct bacterial community succession patterns over the observed time, and elucidated the fate of oil-derived fallout on the seafloor.

3.3 Materials and Methods

Sampling: Our sediment samples were obtained from five cruises (Figure 18, Table 7). The earliest sediment samples collected during the DWH blowout (May 5-8, 2010) were obtained during a Rapid Response cruise on R/V *Pelican* by boxcorer ca. 3 miles northwest (PE6) and 2.7 miles southeast (PE21) of the wellhead, respectively; they were free of gas smell and oily flocs. About two months after the Macondo wellhead had been capped on July 15, 2010, oil-contaminated sediments were collected by RV *Oceanus* (Aug. 21 – Sept 16, 2010) 14 miles northeast (sample C40) and 21 miles west (sample C75) and 22 miles southwest (sample C82). The olive-grey seafloor sediment was covered with a dark red-brown fluffy layer that included oil flocs, identified by green fluorescence under UV light (Figure 19). This surface layer was found continuously in most samples collected during the fall of 2010 (October 10-21, R/V *Cape Hatteras*; November 7-December 3, R/V *Atlantis*), as well as in summer 2011 (July 2-27, R/V *Endeavor*).

In October 2010, sediment cores from 1.6 miles southwest (GIP16) and 2.4 miles north-to-northeast of the wellhead (GIP24) showed the conspicuous red-brown surface layer where petroleum smell and green fluorescent spots under UV light indicated the presence of oil droplets (Figure 19), in contrast to the control sample (GIP08) ca. 57 miles south of the wellhead. In November 2010 oil-contaminated sediment (MUC19) with the red-brown surface layer was obtained near the wellhead, and close to the May 2010 sample PE21. Although a light oil sheen on the supernatant was confirmed by observation under UV light, no smell could be detected. Sediment core MUC20 was taken 17.4 miles south-southeast of the wellhead as a control sample to MUC19; the core was evenly light brown-grey colored, and did not have a red-brown surface

layer. In July 2011, sediments cores E01801 and E01804, ca. 2 miles southeast of the Macondo wellhead, were collected with R/V *Endeavor*, to monitor the long-term dynamics of the sediment microbial community structure. The top red-brown sediment layer was observed again, but lacked petroleum smell entirely. In each cruise, control samples without the conspicuous red-brown layer were taken as references; the May 2010 samples provided the pre-sedimentation control. All the sediment cores were sectioned right after retrieval and stored immediately at -80 °C until further molecular processing.

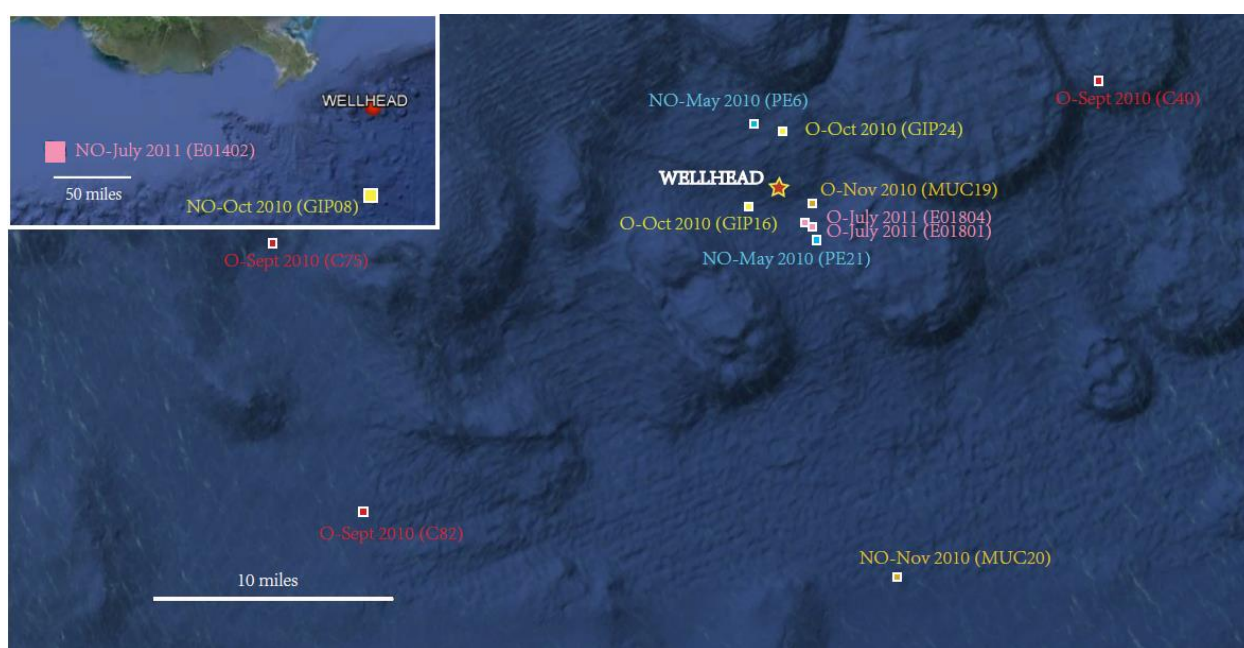


Figure 18. Site map of sediment samples collected from May 2010 to July 2011. Sample names start with O: oily sediment or NO: non-oily sediment, followed by sampling time, and samples ID in parenthesis.

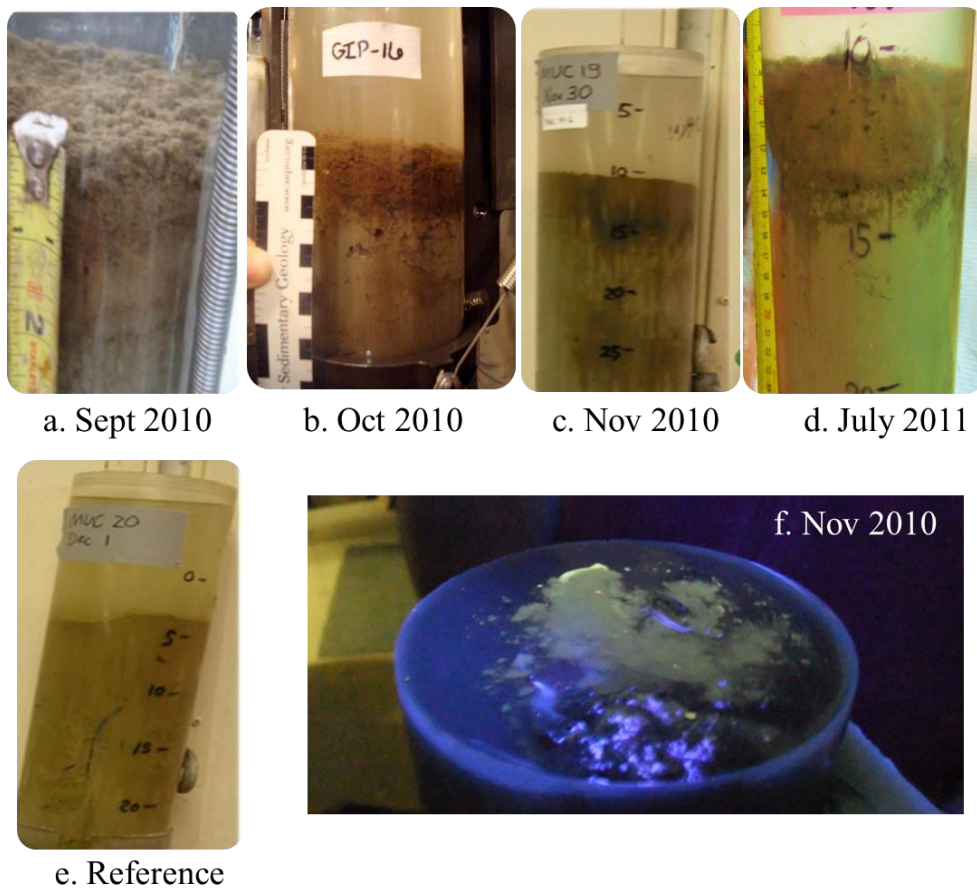


Figure 19. Oil contaminated sediment cores, with a fluffy red-brown layer contains oil aggregates sitting at the top of the gray sediment. a-d: oil-contaminated cored recovered from the wellhead area at different time points. e: a reference core from 15 miles south of the wellhead. f: under UV light, top of the Nov 2010 core fluoresce indicating the existence of oil.

Sample names	Ship	Date	Depth (m)	Latitude (N)	Longitude (W)	Oil polluted (Y/N)	Sample layer
PE6	RV <i>Pelican</i>	5/5/2010	1380	28°46.557	88°24.293	N	0-3 cm
PE21	RV <i>Pelican</i>	5/8/2010	1605	28°42.150	88°21.729	N	0-3 cm
C40	RV <i>Oceanus</i>		1496	28°47.282	88°10.020	Y	0-3 cm
C75	RV <i>Oceanus</i>		1087	28°42.650	88°44.900	Y	0-1 cm
C82	RV <i>Oceanus</i>		1372	28°32.950	88°40.760	Y	0-1 cm
GIP24	RV <i>Cape Hatteras</i>	10/17/2010	1418	28°46.235	88°22.874	Y	0-1 cm
GIP16	RV <i>Cape Hatteras</i>	10/16/2010	1560	28°43.383	88°24.577	Y	0-1 cm
GIP16 (RNA)	RV <i>Cape Hatteras</i>	10/16/2010	1560	28°43.383	88°24.577	Y	0-1 cm
GIP16 3-4 cm	RV <i>Cape Hatteras</i>	10/16/2010	1560	28°43.383	88°24.577	N	3-4 cm
GIP08	RV <i>Cape Hatteras</i>	10/13/2010	2360	27°54.370	88°27.001	N	0-1 cm
GIP08(RNA)	RV <i>Cape Hatteras</i>	10/13/2010	2360	27°54.370	88°27.001	N	0-1 cm
MUC19	RV <i>Atlantis</i>	11/30/2010	1574	28°43.350	88°21.770	Y	0-2.5 cm
MUC20	RV <i>Atlantis</i>	12/1/2010	1885	28°29.290	88°19.050	N	0-2.5 cm
E01801	RV <i>Endeavor</i>	7/25/2011	1630	28°42.382	88°21.815	Y	0-2 cm
E01804	RV <i>Endeavor</i>	7/26/2011	1620	28°42.491	88°21.999	Y	0-2 cm
E01402	RV <i>Endeavor</i>	7/21/2011	64	28°20.919	91°49.563	N	0-2 cm

Table 7. Samples collected on multiple research cruises near the Macondo wellhead with dates, depths, and geographical coordinates.

DNA and RNA extraction: Only the top surface layer from each sediment sample was used for DNA and RNA extraction (Table 7). Total sediment DNA was extracted from 0.25 g of each sample by using the PowerSoil™ DNA isolation kit (MOBIO Laboratories, Inc.), according to the manufacturer's instruction manual. Sediment for RNA extraction was thawed in trichloroacetic acid (TCA) lysis buffer, bead beaten, and nucleic acids were precipitated in 0.6 volume isopropanol overnight at -20 °C (McIlroy et al., 2008). Precipitated nucleic acids were resuspended in water and extracted via a sequence of multiple separations with low-pH (5.1) phenol, phenol-chloroform, and chloroform, and subsequently precipitated overnight at -20 °C in 0.7 by volume isopropanol and 0.5 by volume ammonium acetate (Lin et al., 1995; MacGregor et al., 1997). Nucleic acid pellets were resuspended in nuclease free water, and purified using the RNeasy RNA cleanup kit (Qiagen, Germantown, MD). DNase treatments, using Turbo DNase I (Thermo Fisher Scientific, Waltham, MA) either on the column during RNeasy cleanup or in solution or both, were necessary to eliminate PCR-detectable DNA.

PCR amplification and cloning. The resuspended rRNA was reverse transcribed to cDNA by the SuperScript® III One-Step RT-PCR system with Platinum® Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA) according to the recommended reaction conditions. Then the cDNA and the extracted total DNA were amplified with Speedstar DNA polymerase (TaKaRa, Shiga, Japan) using the bacterial primers 8F and 1492R (Teske *et al.*, 2002) and the manufacturer's recommended concentration for buffer, dNTPs and DNA polymerase. Each PCR reaction consisted of 2 µl DNA extract, 2.5 µl 10x FBI buffer (TaKaRa, Shiga, Japan), 2.0 µl dNTP mix, 2.0 µl 10 µM solution of primers 8F and 1492R (Invitrogen, Carlsbad, CA), respectively, 0.25 µl SpeedStar polymerase (TaKaRa), and was brought to 25 µl with sterile H₂O. Amplification was performed in a BioRad iCycler Thermal Cycler (Hercules, CA) as follows: initial denaturation at 95 °C for 4 minutes, 25 cycles of 95 °C (10 seconds), 55 °C (15 seconds) and 72 °C (20 seconds), and a final 10 minute extension of 72 °C. PCR and RT-PCR product aliquots, including positive and negative controls, were SYBR green stained and visualized using a 1.5% agarose gel. The products were purified using the MinElute® PCR Purification Kit according to the manufacturer's instructions (Qiagen, Valencia, CA), and cloned into OneShot® TOP10 competent Cells (Invitrogen, Carlsbad, CA) using the TOPO TA Cloning® Kit for Sequencing (Invitrogen) according to the manufacturer's instructions. Transformed cells were grown on LB/Xgal/Kanamycin plates. Individual white colonies were arbitrarily picked, re-plated and sanger-sequenced at Genewiz Corporation (South Plainfield, NJ) using vector primers M13 F and M13 R.

Phylogenetic Analysis. Near-complete 16S rRNA gene sequences were analyzed using Sequencher (Gene Codes, Ann Arbor, MI) and compared to other sequences via the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information

(<http://blast.ncbi.nlm.nih.gov/>) (Altschul *et al.*, 1990). Chimera check was performed by using Pintail 1.1 software (Ashelford *et al.*, 2005). After construction of a general 16S rRNA alignment using the ARB phylogeny software package (Ludwig *et al.*, 2004) and the SILVA v95 database (Pruesse *et al.*, 2007), separate alignments for the *Gamma*- and *Alphaproteobacteria* were prepared with sequences for related *Gammaproteobacteria* and *Alphaproteobacteria*. Sequences of well-characterized pure cultures and described species were used for phylogenies whenever possible; otherwise, molecular phylotypes with an informative literature history were selected to anchor major phylogenetic branches of uncultured bacteria. Phylogenetic trees were constructed and bootstrap checks (1000 reruns) of the tree topology were performed using ARB's neighbor-joining function with Jukes-Cantor correction.

Statistical analysis. To compare the homogeneity of the sediment 16S rRNA and rDNA clone libraries, PCA hierarchical clustering was performed by using UniFrac online program <http://bmf.colorado.edu/unifrac/> (Lozupone *et al.*, 2011) using a NJ tree generated with ARB software. X-Fig and Adobe Illustrator CS5.5 were used to edit image files and figures when necessary.

3.4 Results and Discussion

3.4.1 Bacterial community change through timeline at phylum/sub-phylum resolution

The time series survey of bacterial community structure in the wellhead area sediment revealed a complex pattern of the microbial community succession. The two samples (PE6 and PE21) collected in early May 2010 were free of oily flocs at the sediment surface and lacked any petroleum smell, indicating that oil-derived precipitation had not yet settled on the seafloor. Therefore, PE6 and PE21 are considered as pre-sedimentation control samples that provide the baseline for this study. The 16S rRNA gene clone libraries indicated a sediment bacterial community where a complex gammaproteobacterial assemblage, uncultured deltaproteobacterial

lineages USD4 (unsolved division 4), and members of the phyla *Bacteroidetes*, *Planctomycetes* and *Actinobacteria* together accounted for more than half (55.5% in PE6 and 74.1% in PE21, respectively) of all clones (Figure 20). PE6 and PE21 also shared as minority groups *Betaproteobacteria*, *Alphaproteobacteria*, *Acidobacteria*, *Gemmatimonadetes*, *Nitrospira*, and *Chloroflexi*. Members of the phyla *Verrucomicrobia* and *Firmicutes* were only found in PE6 but not in PE21.

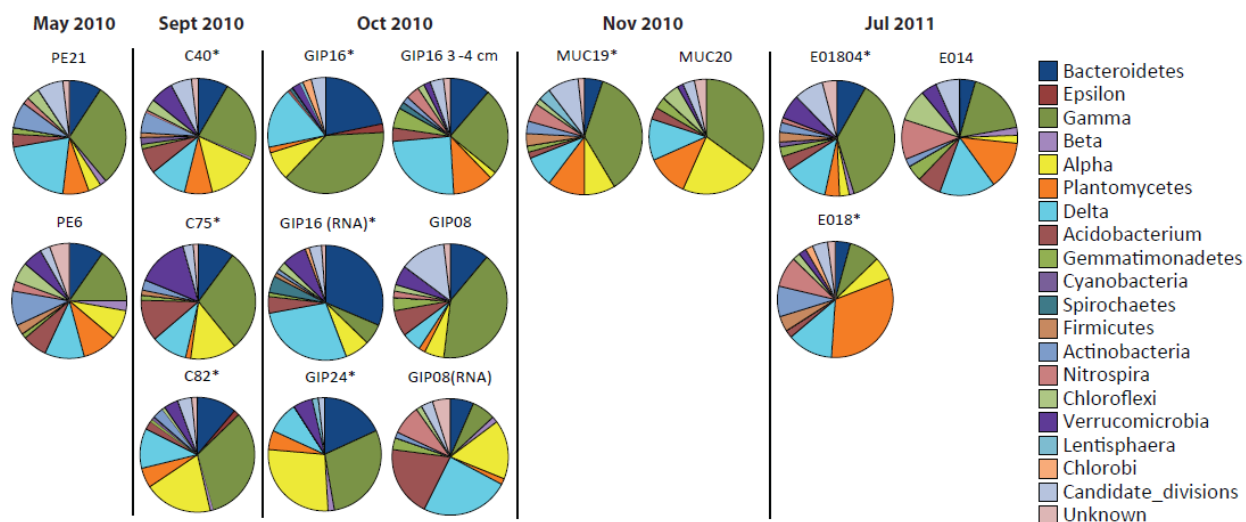


Figure 20. Pie chart comparison of bacterial 16S rRNA gene clone libraries and reverse-transcript 16S rRNA clone libraries from oil polluted samples (labeled with *) and control sites. The bacterial communities are shown at phylum-level resolution.

Oil-impacted sediment cores were recovered during the R/V *Oceanus* cruise in August/September 2010 (Joye *et al.*, 2014). Strongly depleted $\delta^{14}\text{C}$ and $\delta^{13}\text{C}$ values, and high Th-234 and Pb-210 activity showed that the red-brown surface layer of these cores was petroleum-originated and had precipitated recently (Chanton, 2013, Brooks *et al.*, 2014). Hydrocarbon fingerprint spectra confirmed the source of this layer was the Macondo oil (Joye *et al.* unpublished data). The 16S rRNA gene survey demonstrated that the surficial sediment bacterial communities changed in response to the oil input; in all three samples (C40, C75 and

C82), *Alphaproteobacteria* increased into the second most abundant group after *Gammaproteobacteria*, and *Actinobacteria* decreased and became a minority group; other major groups showed little change. Among the minor groups, *Verrucomicrobia* and *Acidobacterium* increased in some samples (Table 8).

	May 2010		Sept. 2010			Oct. 2010 oil-polluted			Oct. 2010 non-oil			Nov. 2010		July 2010		
	PE6	PE21	C40	C75	C82	GIP24	GIP16	GIP16(RNA)	GIP16 3-4 cm	GIP08	GIP08(RNA)	MUC19	MUC20	E01801	E01804	E01402
Bacteroidetes	9.7%	9.3%	8.4%	10.1%	11.2%	18.2%	21.8%	31.4%	11.3%	11.1%	6.6%	5.2%	0.0%	4.3%	8.2%	4.4%
Epsilonproteobacteria	0.0%	0.0%	0.0%	0.0%	1.6%	0.0%	2.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Gammaproteobacteria	15.3%	29.6%	22.7%	29.0%	32.8%	29.1%	37.9%	5.8%	24.5%	40.7%	6.6%	36.2%	35.0%	8.5%	37.0%	17.8%
Betaproteobacteria	2.8%	1.9%	0.6%	0.0%	0.8%	1.8%	0.0%	0.0%	0.0%	0.0%	1.6%	0.0%	0.0%	0.0%	1.4%	2.2%
Alphaproteobacteria	8.3%	3.7%	14.3%	13.0%	19.2%	27.3%	8.1%	7.0%	1.9%	5.6%	16.4%	8.6%	21.7%	6.4%	2.7%	2.2%
Planctomycetes	9.7%	7.4%	7.8%	1.4%	5.6%	5.5%	1.6%	0.0%	11.3%	1.9%	1.6%	10.3%	11.7%	31.9%	4.1%	13.3%
Deltaproteobacteria	11.1%	20.4%	10.4%	10.1%	11.2%	9.1%	16.9%	27.9%	24.5%	5.6%	24.6%	8.6%	11.7%	12.8%	12.3%	15.6%
Acidobacterium	6.9%	3.7%	7.1%	11.6%	2.4%	0.0%	0.8%	4.7%	3.8%	7.4%	19.7%	1.7%	3.3%	2.1%	4.1%	6.7%
Gemmatimonadetes	1.4%	1.9%	1.3%	1.4%	0.8%	0.0%	0.0%	1.2%	5.7%	3.7%	3.3%	1.7%	3.3%	0.0%	2.7%	4.4%
Cyanobacteria	0.0%	0.0%	1.9%	0.0%	0.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	1.4%	0.0%
Spirochaetes	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.8%	4.7%	1.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Firmicutes	2.8%	0.0%	1.3%	1.4%	0.0%	0.0%	0.0%	1.2%	0.0%	0.0%	0.0%	3.4%	0.0%	4.3%	2.7%	0.0%
Actinobacteria	9.7%	7.4%	5.8%	2.9%	3.2%	0.0%	0.0%	1.2%	1.9%	0.0%	1.6%	3.4%	0.0%	8.5%	2.7%	2.2%
Nitrospira	2.8%	1.9%	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	3.8%	1.9%	8.2%	5.2%	0.0%	8.5%	1.4%	11.1%
Chloroflexi	5.6%	3.7%	3.2%	0.0%	0.8%	0.0%	0.0%	2.3%	1.9%	1.9%	1.6%	1.7%	5.0%	2.1%	0.0%	8.9%
Verrucomicrobia	5.6%	0.0%	6.5%	14.5%	4.0%	5.5%	2.4%	7.0%	1.9%	5.6%	0.0%	0.0%	1.7%	2.1%	6.8%	4.4%
Lentisphaera	0.0%	0.0%	0.0%	0.0%	0.0%	1.8%	0.8%	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%	0.0%	0.0%	0.0%
Chlorobi	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.4%	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%	2.1%	0.0%	0.0%
Candidate_divisions	2.8%	7.4%	5.8%	2.9%	4.0%	1.8%	4.0%	3.5%	3.8%	13.0%	3.3%	8.6%	3.3%	4.3%	8.2%	6.7%
Unknown	5.6%	1.9%	1.9%	1.4%	1.6%	0.0%	0.0%	1.2%	1.9%	1.9%	4.9%	1.7%	3.3%	2.1%	4.1%	0.0%

Table 8. Percentage of main phyla in 16 sediment bacterial 16S rRNA and rRNA gene clone libraries from May 2010 to July 2011. The libraries marked as gray indicate oil-polluted samples, and control sites are not marked. The dramatic change of the dominant phyla was marked in the rectangles and was discussed in the text.

One month later, more oil-contaminated cores were recovered during the October 2010 R/V *Cape Hatteras* cruise from the wellhead area. The 16S rRNA gene clone libraries of oil-contaminated samples GIP16 and GIP24 showed that the clone library contribution of the phylum *Bacteroidetes* (21.8%, 18.2%) almost doubled in October compared to the May and September samples (Table 8). *Deltaproteobacteria*, especially the strictly anaerobic sulfate-reducing bacteria (SRB), increased sharply in GIP16 (16.9%), but remained near September levels (9.1%) in GIP24. While *Alphaproteobacteria* decreased in GIP16, they constituted the second-most frequently detected group in GIP24 (27.3%). The reference core GIP08 had the highest proportion of *Gammaproteobacteria* (40.7%) of all samples.

The detection of SRB bacteria lineages is consistent with metagenomic and geochemical evidence for oil-stimulated anaerobic metabolisms in the sediment surface. Increased abundance of denitrification genes in oil-contaminated surficial sediments (Mason *et al.*, 2014), increased deposition of manganese oxide due to enhanced metal cycling in surficial sediment near the wellhead (Hastings *et al.*, 2014), and increased occurrence of *Deltaproteobacteria* and anaerobic functional genes 1.5 to 3 cm below the sediment surface (Kimes *et al.*, 2013) show that nitrate-reducing, metal-cycling and sulfate-reducing microbial communities coexist within the oil-impacted surficial sediments, and must have been stimulated by increased hydrocarbon availability.

The question arises whether the original seafloor microbial community persists underneath these oil-impacted surface sediments and their altered microbial communities. To check this hypothesis, we sequenced the 3-4cm olive-grey sediment layer of core GIP16, right below the red-brown surface sediment. The GIP16 3-4cm library diverged from the surface layer results but resembled the libraries of the pre-spill samples PE6 and PE21 in phylum composition, indicating that the original benthic community persists to some extent underneath the oil-impacted layer. The GIP16 3-4cm library shared 13 and 12 phyla and sub-phyla with PE6 and PE21, respectively, compared to 9 groups shared with the GIP16 surface layer. The GIP16 3-4cm and PE6 and PE21 libraries, but not GIP16, shared low proportion of *Bacteroidetes* and a high percentage of *Planctomycetes*. The GIP16 3-4cm library and the October reference library GIP08 shared 12 groups together, 7 of which were represented by less than 8% each (Figure 20, Table 8). *Planctomycetes* and *Deltaproteobacteria* appeared in GIP08 in lower abundance of 1.9% and 5.6%, compared to 11.3% and 24.5% for GIP16 3-4cm.

To identify the active members in the changing bacterial community structure in October sediments, reverse-transcribed 16S rRNA clone libraries were prepared from surface sediment of GIP16 and the GIP08 control sediment. At the phylum level, *Deltaproteobacteria* remained important; they accounted for the second dominant group in GIP16 (27.9%) and the top group (24.6%) in GIP08. *Bacteroidetes* followed the same trend in the reverse-transcribed 16S rRNA clone libraries as in the 16S rRNA gene libraries; high abundance was observed in GIP16 but low in GIP08. *Gammaproteobacteria*, the dominant group in most DNA-based sediment 16S rRNA gene clone libraries, contributed much less in the reverse-transcribed 16S rRNA clone libraries of GIP16 and GIP08 (6.6%, 5.8%). The GIP08 reverse-transcribed 16S rRNA library diverged from its GIP16 counterpart due to large proportions of *Acidobacterium* (19.7%) and *Alphaproteobacteria* (16.4%) (Figure 20, Table 8). Most minor groups in the 16S rRNA gene libraries appeared in low percentages in both rRNA clone libraries.

The bacterial communities of cores MUC19 and MUC20, taken in late November 2010, resembled each other on the phylum level. *Gammaproteobacteria* dominated with 36.2 and 35.0%; *Alphaproteobacteria* were high but variable (8.6 and 21.7%); *Deltaproteobacteria* were abundant but did not reach the peak of GIP16 (8.6 and 11.7%); and *Planctomycetes* were conspicuously elevated (10.3 and 11.7%) compared to October and September samples, and are closer to the May PE samples. This partial realignment in phylum-level composition and relative abundance for both oil-impacted (MUC19) and control core (MUC20) towards each other and the May 2010 PE samples, suggests decreasing oil impact on community structure (Figure 20, Table 8).

In July 2011, one year after the wellhead was capped, *Planctomycetes* that had already rebounded in the November 2010 samples overtook *Gammaproteobacteria* as the most dominant

proportion of the 16S rRNA gene clone library (31.9%) of oil-impacted core E01801, whereas the *Gammaproteobacteria* dominated in nearby core E01804 (37.0%) and *Planctomycetes* accounted only for 4.1 % (Figure 20). These distinct bacterial community structure results in two adjacent oil-impacted cores collected near the Macondo wellhead one year after the DWH blowout may indicate patchy contamination of the seafloor by complex oil precipitation patterns, or sediment resuspension by deep currents and redeposition at the seafloor; proximity to the wellhead is not sufficient to push bacterial community succession into a single direction. The reference core E01402 from a shallow near-coastal site (depth 64 m) 211 miles west of the wellhead, has a unique clone library with unusually high proportions of *Nitrospira* and *Chloroflexi* that did not occur in deep water cores (Figure 20).

A complex pattern of microbial community succession within the oil impacted surface seafloor emerged after principal coordinates analysis (PCA) based on phylogenetic affiliations obtained with ARB reconstructions (Ludwig *et al.*, 2004, Lozupone *et al.*, 2006). Principal components PC1 and PC2 explained 32.42% of the variation in bacterial community composition, and PC1 separated most oil-impacted samples (PC1 value > 0) from the non-impacted control samples (Figure 21). The 2010 September oily samples (C40, C75, C82) together with the 2011 July oily sample E01804, and GIP16 and GIP24 formed two separate clusters that approach each other at GIP24 and C82, suggesting a successional change of their DNA-based bacterial community structures from September to October. The 16S rRNA reverse-transcribed clone library of oily sediment (GIP16_RNA) diverged from other clone libraries, and remained least dissimilar to the GIP16 rRNA gene clone library. The non-contaminated DNA samples grouped together, except the near-shore sample E01402 and the 16S rRNA reverse-transcribed clone library of the non-oily sediment GIP08. PCA supports the hypothesis that the

3-4 cm layer underlying oil-impacted surface sediment (GIP16_3_4cm) was previously the non-contaminated seafloor surface, since it clusters with surface samples from uncontaminated sediments, including the May samples PE6 and PE21, the October sample GIP08 and the November sample MUC 20. Interestingly, the November oily (MUC19) and non-oily (MUC20) sediments grouped adjacent to each other and to other non-contaminated samples, indicating decreasing bioavailability and microbial impact of the hydrocarbon fallout. Implicitly, the small amount of fresh oil visible as oil sheen on the top of core MUC19 must have recently been released, and does not represent weathered oil-derived fallout. The 2011 June oily core E01801 was separated from all other samples, most likely by its high abundance of *Planctomycetes*.

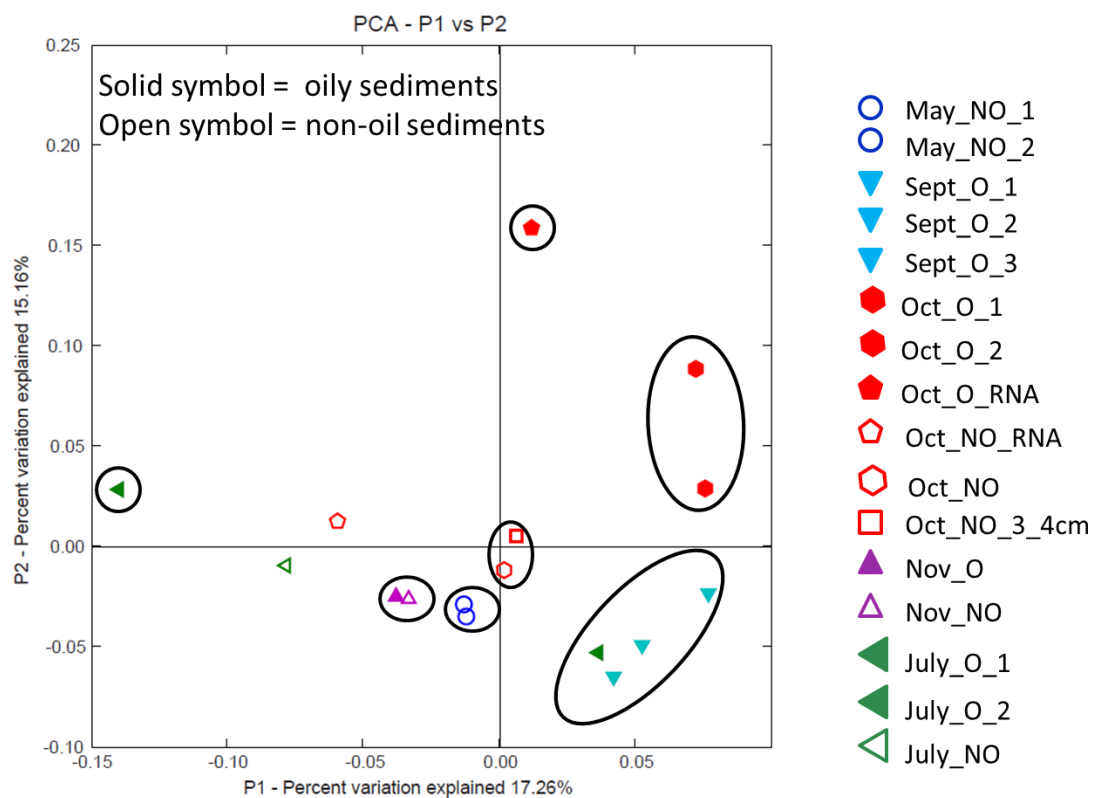


Figure 21. PCA principal component analysis of distances of distances between phyla/sub-phyla level phylotypes in all sediment samples. Samples were labeled by time of collection, oily (O) or non-oily (NO) and samples characteristics.

3.4.2 Bacterial community change through timeline at order/family/genus resolution

Since the classification at phylum/sub-phylum level is too general to suggest physiological properties and functional roles in oil degradation, we analyzed the 16S rRNA gene sequences of the 6 most dominant groups, the *Alphaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, *Bacteroidetes*, *Planctomycetes* and *Verrucomicrobia*, at the level of order, family or genus.

***Alphaproteobacteria*.** Six major subgroups of *Alphaproteobacteria* were detected in this sediment survey (Figure 22). The marine *Roseobacter* clade was not found in samples that preceeded oil-derived sedimentation on the seafloor (PE6 and PE21), but increased on the seafloor in September (C40, C75, C82) and October (GIP24) 2010, strongly suggesting that this group was closely connected to the input of oil-derived marine aggregates. Deep sequencing the partial V4 region of the 16S rRNA gene identified a significant increase of the *Rhodobacteraceae* family on sediment samples with high TPH concentrations (Mason *et al.*, 2014); we suspect this increase is mostly driven by the marine *Roseobacter* clade. The marine *Roseobacter* clade is well represented in diverse habitats, especially in coastal zones and polar regions, in marine snow, phytoplankton, sea ice, sea water and sediments, as well as hydrothermal vents (Wagner-Dobler & Biebl, 2006). The *Roseobacter* phylotypes in the oil polluted sediments may have been enriched in the oil-derived sinking aggregates, as indicated by their prominence in laboratory-generated oil snow particles (Arnosti *et al.*, 2014), or they may represent autochthonous *Roseobacter* in the sediment that were induced and enriched by the oil fallout. *Roseobacter* genomes indicate potential for carbon monoxide oxidation, sulfur oxidation, dimethylsulfoniopropionate demethylation, aromatic compound degradation, denitrification and phosphonate utilization (Buchan *et al.*, 2005), consistent with physiological traits of cultured

representatives (Wagner-Dobler & Biebl, 2006). *Roseobacter* isolates could be cultured with media amended with pyrene, naphthalene, fluoranthrene, or phenanthrene (Brito *et al.*, 2006, Pinyakong *et al.*, 2012), and *Roseobacter* populations were enriched in decane, hexadecane and other alkane-degrading seawater microcosms (McKew *et al.*, 2007); they should therefore play a role in alkane and PAH degradation. The cold in-situ temperatures (4°C) at the Gulf of Mexico seafloor are compatible with oil degradation by *Roseobacter* (Coulon *et al.*, 2007).

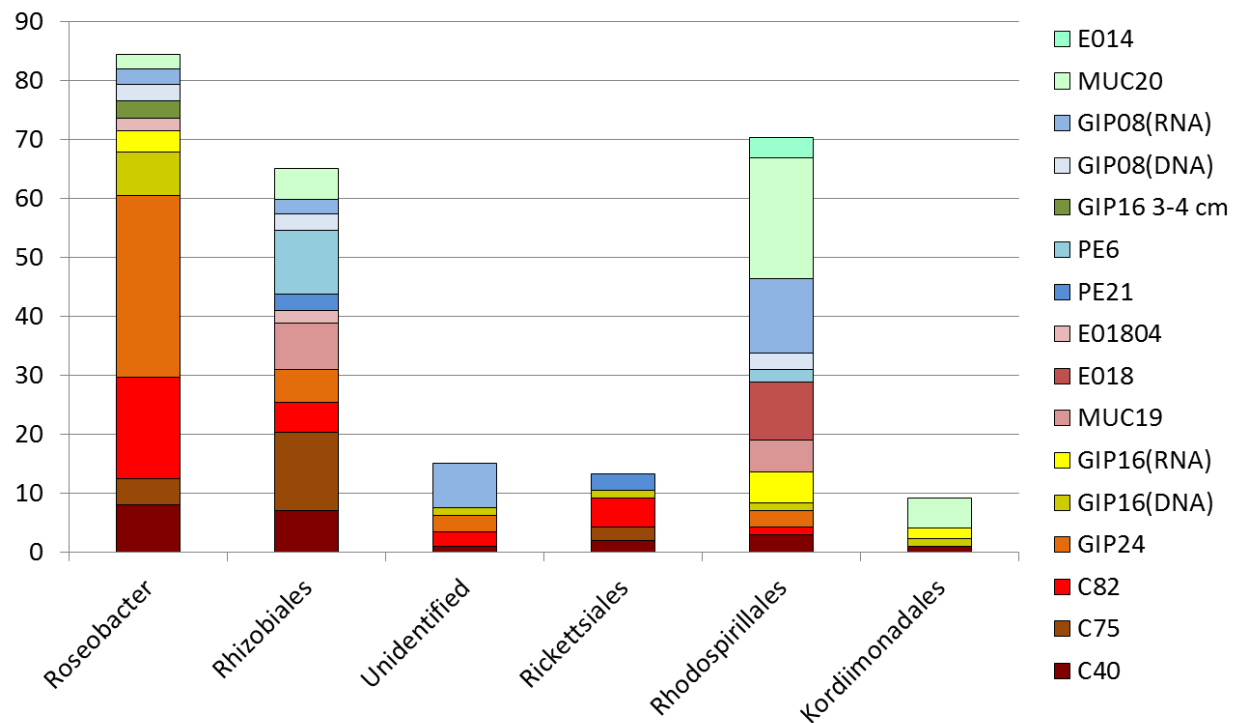


Figure 22. Alphaproteobacterial community change throughout the study time based on order/family/genus resolution

Deltaproteobacteria. The sulfate-reducing bacterial families *Desulfobulbaceae* and *Desulfobacteraceae*, including the *Desulfosarcina/Desulfococcus* (DSS) clade, and two uncultured clusters (USD4 and USD5) constitute the principal deltaproteobacterial groups in these sediment samples. The *Desulfobacteraceae* and *Desulfobulbaceae* were mainly represented

by sequences from oil-polluted surficial sediments of site GIP16 (both in 16S rRNA gene clone and reverse-transcript 16S rRNA clone libraries) and GIP24, collected in October 2010 (Figure 23). Members of the DSS group can degrade short chain alkanes under strict anoxic conditions in coastal sediment as well as deep seafloor; the *Desulfobacteraceae* and *Desulfobulbaceae* also contain potentially anaerobic aromatic hydrocarbon degraders (Teske, 2010). Although members of these two families were previously found in natural seeps, mud volcanoes and gas hydrates in the Gulf of Mexico (Lloyd *et al.*, 2010, Orcutt *et al.*, 2010), several lines of evidence indicate that these SRBs found in this study were enriched by oil fallout instead of natural seepage. The GIP16 and GIP24 sediment cores were not obtained from seep sites, since they did not contain abundant porewater methane or sulfide, and were not permeated by up-migrating hydrocarbons. Except for a single clone from the 3-4 cm layer, the *Desulfobacteraceae* were limited to the surface sediment and did not thrive below the surface, contrary to observations in active seeps (Lloyd *et al.*, 2010). Members of the *Desulfobulbaceae* obtained from the GIP16 and 24 sediments were closely related to clones detected in coastal sediments contaminated by the Prestige oil spill (Acosta-Gonzalez *et al.*, 2013) and formed a lineage within the moderately psychrophilic genus *Desulforhopalus*, which is capable of sulfate reduction and elemental sulfur disproportionation (Isaksen & Teske, 1996) (Figure 28). Other deltaproteobacterial subgroups, such as the *Myxococcales*, one of the enriched bacterial groups in oil-polluted sediment after the Prestige oil spill (Acosta-Gonzalez *et al.*, 2013), were not specifically associated with oil-contaminated seafloor sediment in this dataset.

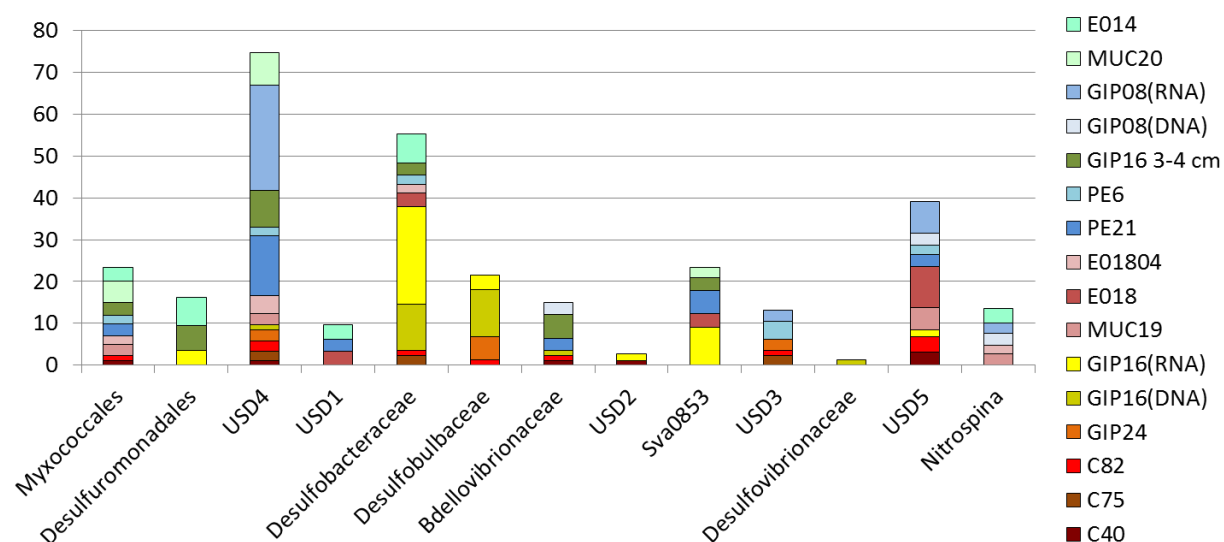


Figure 23. Deltaproteobacterial community change throughout the study time based on order/family/genus resolution.

***Gammaproteobacteria*.** Members of the *Gammaproteobacteria* occurred in high proportions in nearly all clone libraries, except sample E01801 collected in July 2011. Within the *Gammaproteobacteria*, 15 subgroups were identified showing the high diversity of this subphylum. Specifically, members of the obligate aromatic hydrocarbon degrader *Cycloclasticus* appeared in oil-polluted surface sediment sampled in September, October and November 2010. Previously, *Cycloclasticus* had been found in surface oil slick collected in May, during the late stage of the hydrocarbon plume in June, and in the post-plume deep water in September 2010 (Yang *et al.*, 2014), as well as in the marine snow formed in lab incubations (Arnosti *et al.*, 2014), but it was not detected in non-polluted sediments or oily sediments after November 2010 (Figure 24). Pure cultures of *Cycloclasticus* were previously obtained from Gulf of Mexico sediments amended with phenanthrene or naphthalene (Geiselbrecht *et al.*, 1998); new strains were isolated also from the sea surface oil collected in May 2010 (Gutierrez *et al.*, 2013). *Cycloclasticus* phylotypes that closely affiliated to the *Cycloclasticus* sequences from surface

slick also contained sequences from both post plume as well as oil contaminated surficial sediment, indicating that the weathered oil-derived aggregates on the seafloor most likely maintained populations of *Cycloclasticus* spp. that had originally arrived on sinking oil-derived particles (Figure 29).

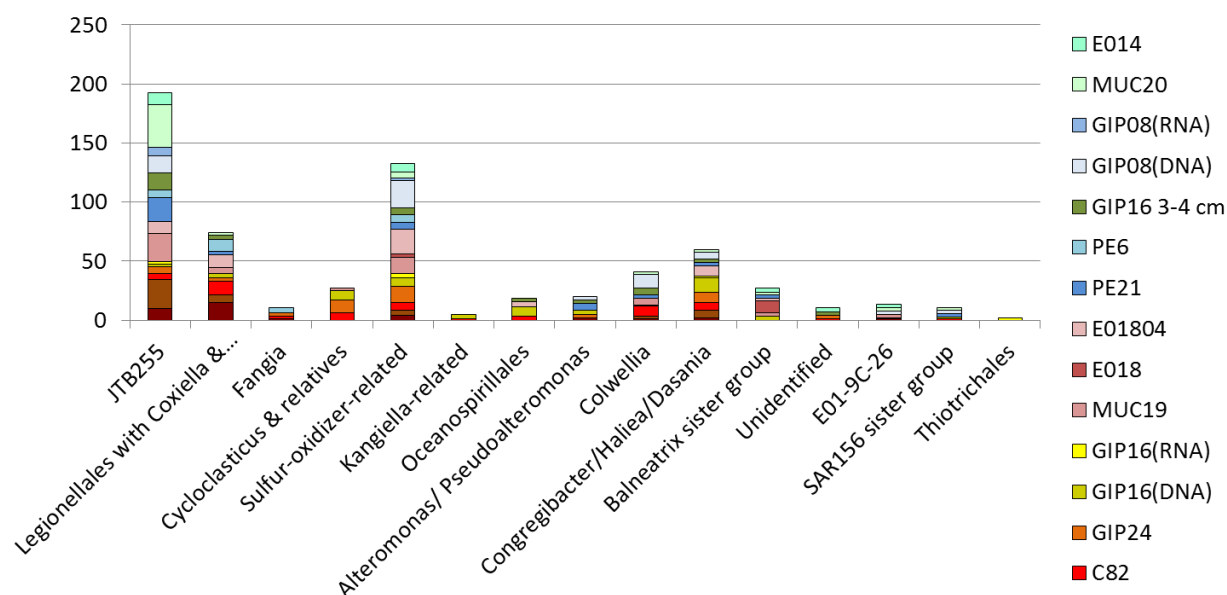


Figure 24. Gammaproteobacterial community change throughout the study time based on order/family/genus resolution

Similar to *Cycloclasticus*, members of the heterotrophic bacterial genus *Colwellia* were previously detected in oil slick, deep hydrocarbon plumes, and post-plume seawater (Valentine *et al.*, 2010, Baelum *et al.*, 2012, Redmond & Valentine, 2012, Yang *et al.*, 2014). *Colwellia* was consistently detected in alkane-amended seawater and isolated from the Gulf of Mexico seawater enrichments supplied with Macondo oil and dispersant Corexit (Baelum *et al.*, 2012). Since *Colwellia* has a wide spectrum of organic substrates, it is not an obligate oil degrader; its habitats include organic-rich marine fish farm sediments (Bissett *et al.*, 2006) and Antarctic continental shelf sediment (Bowman & McCuaig, 2003). In this dataset, *Colwellia* was found in both oily

and non-oily sediments in September, October and November 2010 (Figure 24), suggesting autochthonous sediment populations that are not tied to oil-derived sedimentation. The most abundant gammaproteobacterial group in the deepwater hydrocarbon plume, the DWH Oceanospirillales, were not found in sediments (Yang *et al.*, 2014).

The genera *Cycloclasticus* and *Colwellia* accounted for relatively small portions of the gammaproteobacterial phylotypes; the most abundant group, designated JTB255 was previously found in deep-sea cold seep sediments of the Japan Trench (Li *et al.*, 1999) and diverse marine sediments and basaltic ocean crust (Teske *et al.*, 2011). The oil input apparently did not influence the JTB255 group except in October 2010. A polyphyletic assemblage of sulfur-oxidizer-related bacteria, members of the *Legionellales*, *Coxiella* and *Rickettsia*, and the *Congregibacter/Halilea/Dasania* group constituted the next-most abundant gammaproteobacterial groups, followed by several clades (*Fangia*, a *Kangiella*-related group, *Alteromonas/Pseudoalteromonas*, *Balneatrix* sister group, E01-9C-26 group, SAR156 sister group and *Thiotrichales*) that appeared in smaller proportions (Figure 24).

***Bacteroidetes*.** Within *Bacteroidetes*, the *Flavobacteriaceae* and the *Marinifilum/Cytophaga* group accounted for most of the clone library abundance of this phylum in oil-impacted September and October samples; the rRNA survey also confirmed their high activity at that time (Figure 25). The *Flavobacteriaceae* increased already in the September 2010 oil contaminated sediments, whereas the *Marinifilum/Cytophaga* group was not detected in the September 2010 sediments but peaked one month later. Several studies indicated that members of the *Flavobacteriaceae* and *Cytophaga* responded strongly to the presence of hydrocarbons. In a coastal sediment polluted by the 2002 Prestige oil spill, *Bacteroidetes* was poorly represented in 2004, but had become a major component of the bacterial community in 2007 that was

specifically dominated by *Flavobacteria* (Acosta-Gonzalez *et al.*, 2013). Uncultured *Cytophaga* groups showed subsequent enrichment on crude oil at 5 °C, similar to the temperature at the bottom of Gulf of Mexico (Brakstad & Bonaunet, 2006). Some *Bacteroidetes* showed hydrocarbon biodegradation abilities; the species *Yeosuana aromativorans* from estuarine sediment and seawater is capable of degrading PAHs such as pyrene (Kwon *et al.*, 2006, Sheppard *et al.*, 2012), and *Flavobacterium* sp. isolated from sewage were able to grow on biphenyl (Stucki & Alexander, 1987).

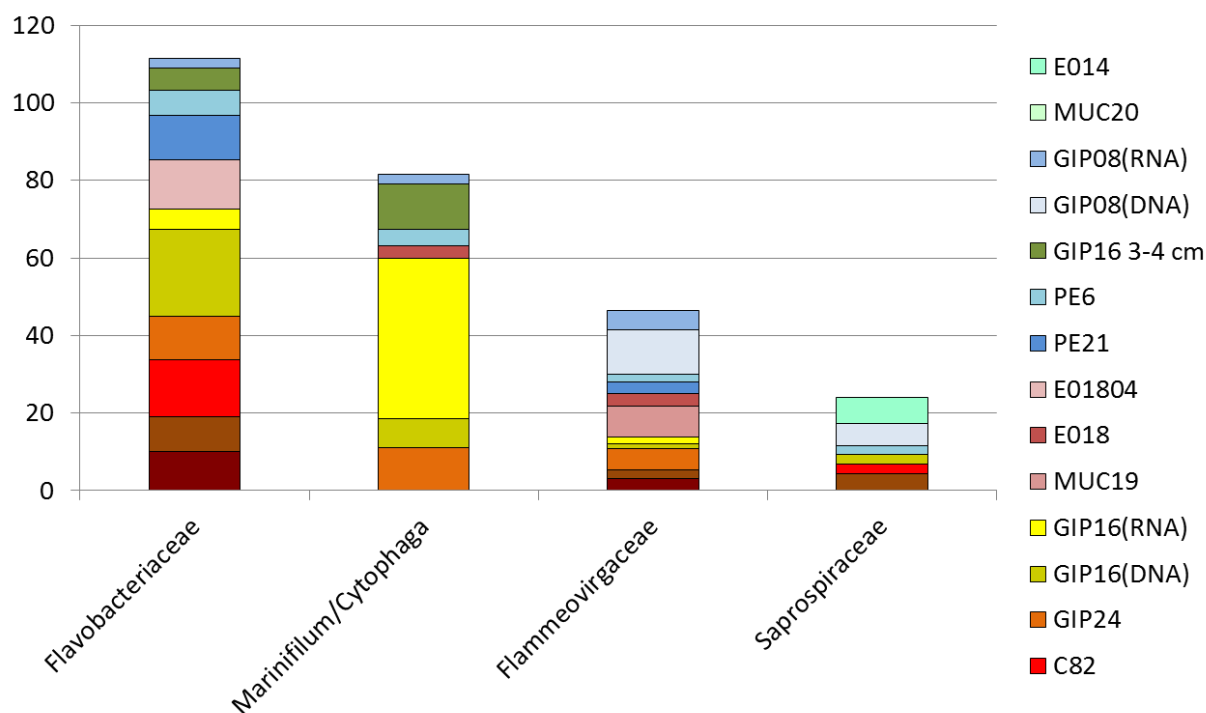


Figure 25. *Bacteroidetes* community change throughout the study time based on order/family/genus resolution

Planctomycetes. Within oil-impacted sediments, the phylum *Planctomycetes* was mostly represented by the clade that includes the algal isolate *Phycisphaera mikurensis* (Fukunaga *et al.*, 2009), and by clades of cultured Planctomyceaceae (Figure 26). Many of our *Planctomycetes* sequences were closely related to those derived from marine seafloor habitats, such as sediments

in the South China Sea (Hu *et al.*, 2010), deep sea sediment underlying two whale falls (Goffredi & Orphan, 2010), and seafloor lavas from the East Pacific Rise (Santelli *et al.*, 2008).

Planctomycetes presumably play a role in carbon recycling in marine environment, even under oxygen-limiting conditions such as in oxygen minimum zones. Their association with marine snow particles (DeLong *et al.*, 1993) and the high numbers of sulfatases in marine

Planctomycetes characterizes them as specialists for the initial breakdown of sulfatated heteropolysaccharides in marine snow and indicate their importance for recycling carbon from these compounds (Woebken *et al.*, 2007). In the seafloor clone libraries, *Planctomycetes* do not show any preference for oil-impacted sediments, except after a delay of one year in the case of sample E018, and in this regard they contrast with other bacterial groups that are stimulated by fresh oil sedimentation. Clones of the Anammox clade (in marine environments, genus *Scalindua*) were mostly obtained from the GIP3-4 cm sample, indicating a potential enrichment in sediments below the oil-derived surface layer.

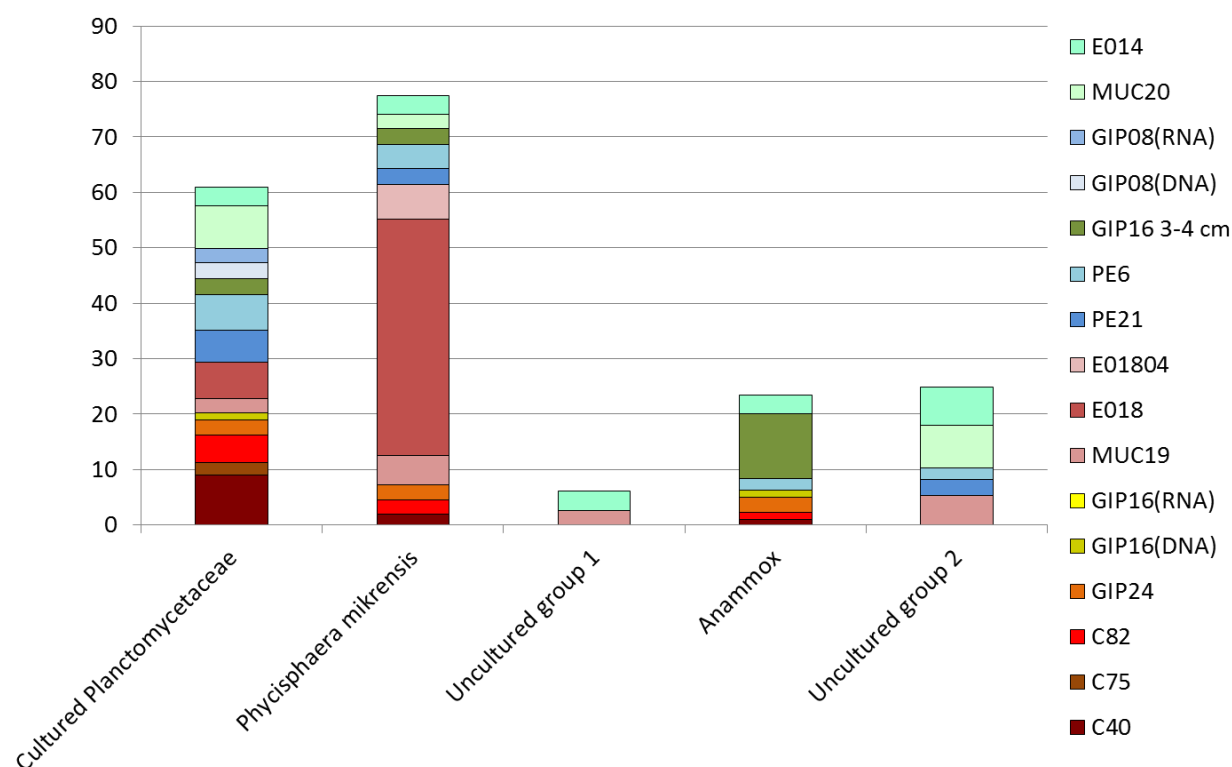


Figure 26. Planctomycetes community change throughout the study time based on order/family/genus resolution

***Verrucomicrobia*.** Although phylotypes of the *Verrucomicrobia* occurred in intermediate abundance in most sediment samples, our family- and genus-level resolution analysis demonstrated contrasting dynamics between the *Verrucomicrobia* subgroups. The phylum *Verrucomicrobia* contains 5 subdivisions but only a handful of pure cultures (Hugenholtz *et al.*, 1998, Freitas *et al.*, 2012). The family *Verrucomicrobiaceae*, previously defined as subdivision 1 (Hugenholtz *et al.*, 1998), contains the methanotrophic genus *Acidomethylosilex* (Freitas *et al.*, 2012) and responded quickly to the oil input in early September 2010, together with the marine *Roseobacter* clade. In contrast, the uncultured subdivision 5 group was highly active in mid-October 2010 (Figure 27). Since representative sequences of subdivision 5 were originally cloned from the methanogenic layer of an aquifer contaminated with hydrocarbons and

chlorinated solvents (Dojka *et al.*, 1998), this subdivision 5 may be capable of bioremediation and survival in anoxic environments.

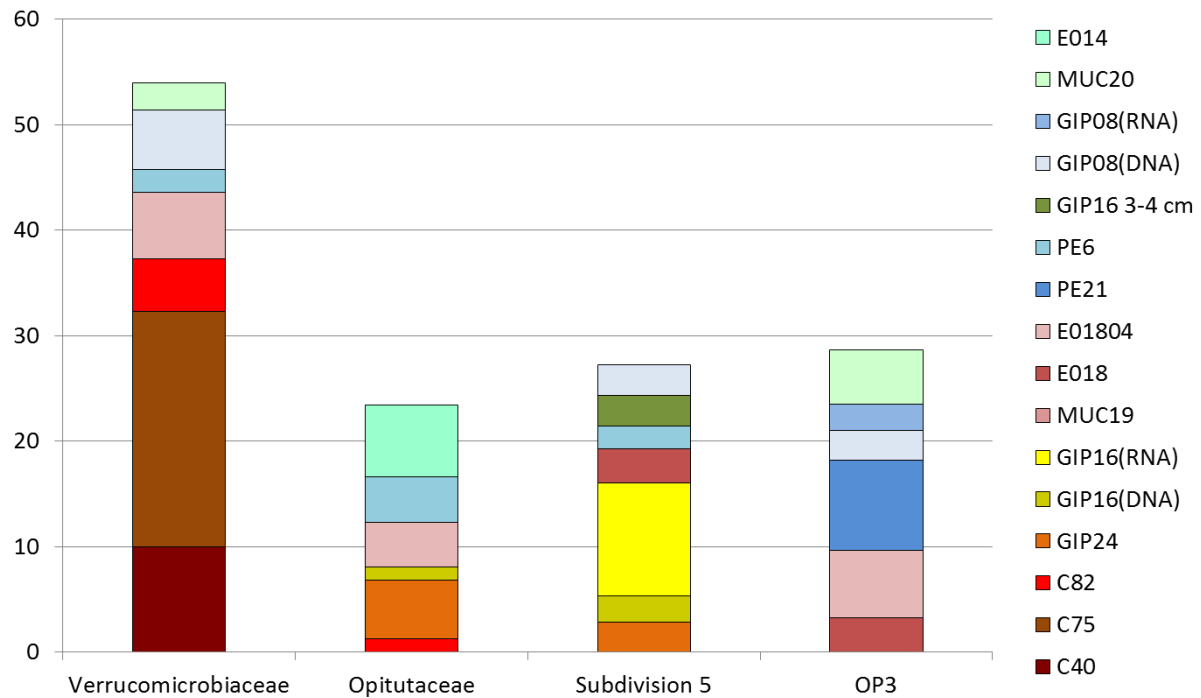


Figure 27. Verrucomicrobial community change throughout the study time based on order/family/genus resolution

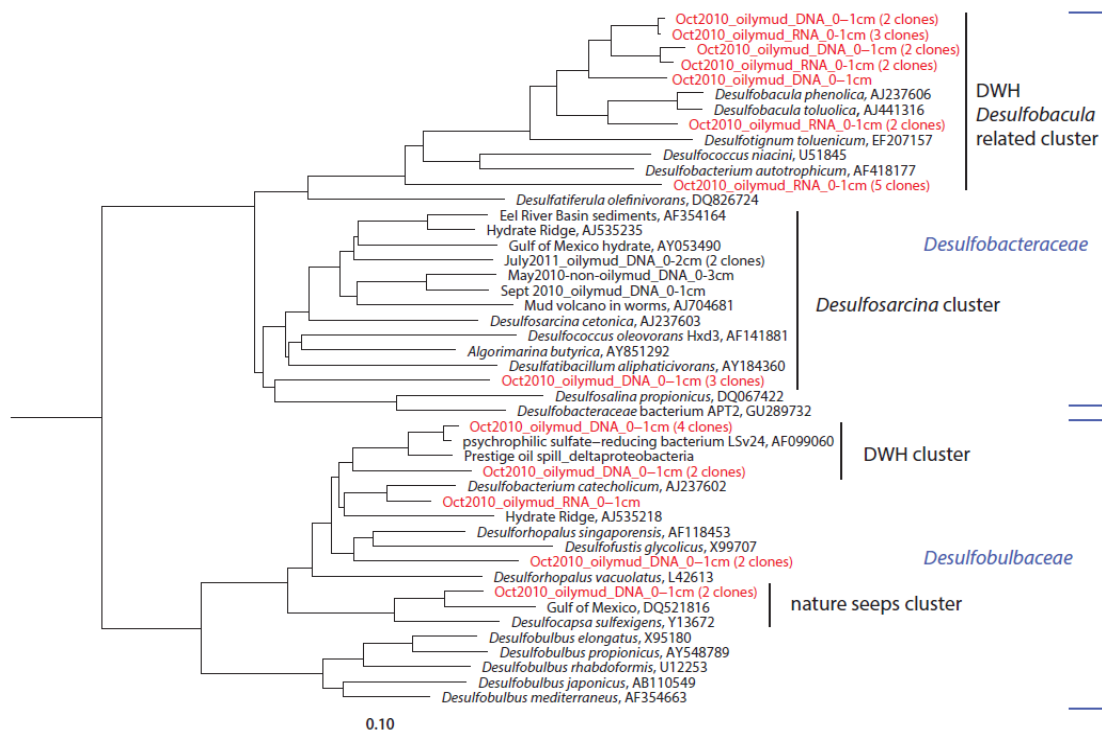


Figure 28. Phylogenetic tree of deltaproteobacterial family *Desulfobacteraceae* and *Desulfobulbaceae*, constructed by sequences from the DWH oil contaminated sediments (red) and from natural seep sediments. Notice that the DWH sequences formed different clusters compared to the natural seep sequences.

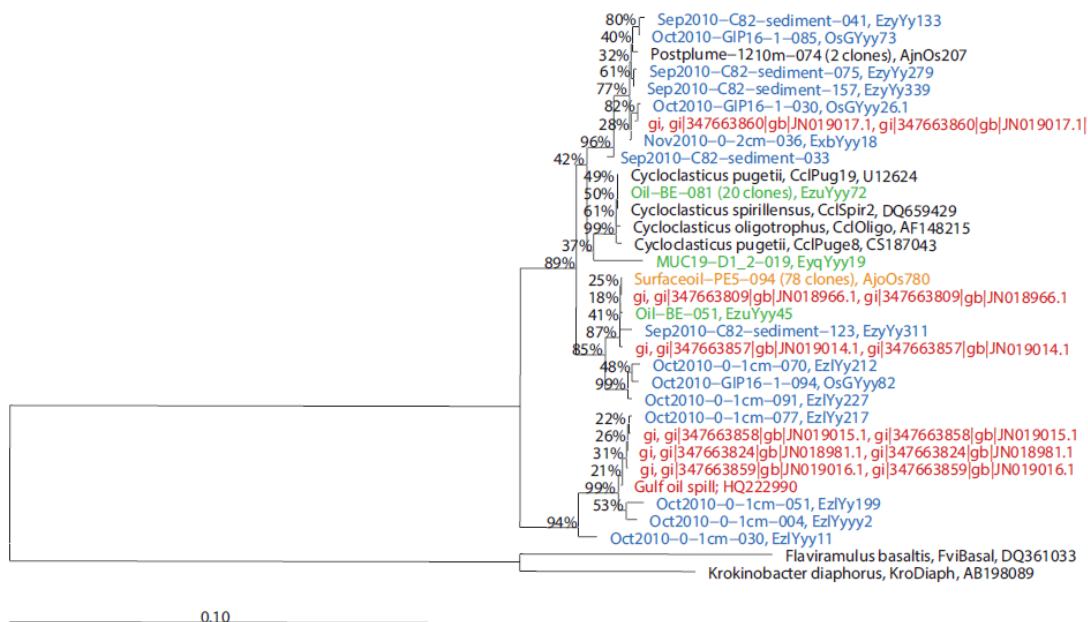


Figure 29. 16S rRNA gene phylogenetic tree of *Cycloclasticus* sequences derived from both water column and the surficial sediment of the Gulf of Mexico at various times. Orange colored clone was from May 2010 surface oil slick; red clones were sequences from post-plume water (Redmond & Valentine, 2012, Valentine et al, 2010); blue clones were from surficial sediments of series time points as labeled; green clones represent sequences derived from the incubated oil slick (chapter 2) or isolated sequences from 4 °C (chapter 4).

3.5 Conclusion

Following the “dirty blizzard” of oil-derived sedimentation on the Gulf of Mexico seafloor, the bacterial community of the oil-impacted seafloor sediments showed dynamic shifts on multiple taxonomic levels during September and October 2010. In these two months, the metabolically versatile *Roseobacter* clade, the sulfate-reducing *Desulfobacteraceae* and *Desulfobulbaceae* within the *Deltaproteobacteria*, the heterotrophic *Flavobacteriaceae* and the *Marinifilum/Cytophaga* group within the *Bacteroidetes*, and the *Verrucomicrobiaceae* within the *Verrucomicrobia* responded strongly to the oil-derived sedimentation pulse; the *Planctomycetes* were selected against by the oil impact in September and October 2010. The coexistence of aerobic and anaerobic bacteria among these sedimentation responders (Mason et al. 2014; this

study) indicates that the surficial oil-impacted sediments provided anoxic microniches; these might have developed as aerobic heterotrophs associated with sedimented marine snow aggregates consumed oxygen locally. By the end of November 2010, the bacterial community structure of oil-impacted sediments had started to realign with those of pre-spill (May 2010) sediments and non-impacted control sediments (Figure 21). This bacterial community trend suggests that the supply of oil-derived substrates had become limiting at this time and no longer supported conspicuous populations of oil-degrading bacterial specialists, as far as detectable with clone libraries. Most likely, high-throughput sequencing methods will detect longer-lasting microbial signatures of the oil-derived seafloor sedimentation. While the long-term ecological damage of the Deepwater Horizon blowout continues to unfold, the microbial early oil spill responders may also be the first indicators of a gradual ecosystem recovery.

CHAPTER 4: HYDROCARBON-DEGRADING BACTERIA ISOLATION AND ENRICHMENT³

4.1 Introduction

Our bacterial 16S rRNA gene clone libraries indicated natural enrichment of the potential hydrocarbon-degrading bacteria, such as uncultured *Oceanospirillales*, *Cycloclasticus*, and *Colwellia* etc. In order to understand the physiology and function of these uncultured oil-degrading bacteria, cultivation was applied to derive pure cultured bacterial strains to determine their premium growth conditions and hydrocarbons they can utilize. However, all the previous attempts of culturing the *Oceanospirillales* enriched in the DWH plume were failed presumably (Mason *et al.*, 2012, Redmond & Valentine, 2012). PAH degrader *Cycloclasticus* species were isolated from the Gulf of Mexico sediment amended with PAHs, indicating the Gulf is a natural habitat to *Cycloclasticus* (Geiselbrecht *et al.*, 1998). A new species of *Colwellia* was derived from the plume seawater amended with the dispersant Corexit under high Fe²⁺ concentration and low temperature (5 °C), but the *Oceanospirillales* grew in the enrichment was not same phylotype as the DWH *Oceanospirillales* (Baelum *et al.*, 2012). In this study, attempts were made to isolate hydrocarbon-degrading bacteria from the surface oil slick, the plume, as well as from the oil contaminated sediment to develop further understanding of the possible bioremediation process and the fate of the released oil in different environments.

³ Partial of this chapter was published as an article, the orginial citation is as follows: Gutierrez T, Singleton DR, Berry D, Yang TT, Aitken MD & Teske A (2013) Hydrocarbon-degrading bacteria enriched by the Deepwater Horizon oil spill identified by cultivation and DNA-SIP. *Isme Journal* 7: 2091-2104

4.2 Materials and Methods

Water column enrichment experiments and isolation. Oil-contaminated surface and plume water samples PE5, B3 and B6 (Table 9) from the Gulf of Mexico oil spill response cruises were used to isolate hydrocarbon-degrading bacteria. 5 µl of surface oil sample (PE5) were streaked directly onto ONR7a agar plates (Dyksterhouse *et al.*, 1995) that were then sprayed with phenanthrene, anthracene, pyrene or fluorine (St Louis, MO, USA) dissolved in acetone (ca. 5% w/v) as the sole source of carbon and energy (Kiyohara *et al.*, 1982). Plume water samples (B3 + B6) were streaked on ONR7a agar plates supplemented with n-hexadecane (1% v/v) (Acros Organics, NJ, USA) as the sole source of carbon and energy. During the preparation of ONR7a medium, the n-hexadecane was added directly to the liquid ONR7a agar after autoclaving. Agar plates were stored in closed plastic bags in the dark at room temperature. Colonies forming clearing zones were picked and subcultured onto fresh ONR7a agar medium amended with the respective PAH until pure cultures were obtained prior to storage in glycerol (30% v/v) at -80 °C.

Sample	Date ^a	Depth (m)	Latitude	Longitude	Distance from wellhead (NM) ^b
During spill:					
Surface (PE5)	May 5	0	28° 44.175 N	88° 22.335 W	0.86
Above plume (B11)	May 30	800	28° 41.686 N	88° 26.081 W	3.5
Within plume (B6)	May 30	1170	28° 41.686 N	88° 26.081 W	3.5
Within plume (B3)	May 30	1210	28° 41.686 N	88° 26.081 W	3.5
Below plume (B1)	May 30	1320	28° 41.686 N	88° 26.081 W	3.5
Post spill:					
Plume (C4B8)	Sep 12	800	28° 41.713 N	88° 26.073 W	3.5
Plume (C4B4)	Sep 12	1210	28° 41.713 N	88° 26.073 W	3.5
Post plume (GIP22)	Oct. 18	1050	28° 40.503 N	87° 39.250 W	37.0

^a Collection date in 2010.

^b NM, nautical miles.

Table 9. Water samples collected from the Deepwater Horizon sites for this enrichment study.

Enrichments with plume water (B3 + B6) were prepared in autoclaved glass test tubes containing 3 ml of liquid ONR7a supplemented with n-hexadecane (5% v/v; ONR + HEX), with series dilutions. The tubes were incubated in the dark with shaking (100 r.p.m.) at 21 °C. After one week, the highest dilutions that yielded growth were selected for dilution-to extinction culturing in ONR + HEX. After repeating this process four more times, samples (5 µl) from the highest dilutions producing growth were streaked onto ONR + HEX agar plates. The plates were stored in the dark at room temperature and after 2 weeks, colonies showing distinct colony morphologies and clearing zones were picked, purified and stored frozen in 30% (v/v) glycerol.

The potential for the strains to degrade phenanthrene or naphthalene was determined using a quantitative spectrophotometric method. For this, acid-washed (0.1N HCl) stream-

sterilized glass test tubes (13 x 100 mm), fitted with screw-caps lined with Teflon-lined silicone septa (Chromacol, Herts, UK) were used. Stock solutions of phenanthrene (ca. 3000 mg l⁻¹) and naphthalene (ca. 9000 mg l⁻¹) dissolved in acetone were prepared. For each strain, two sets of three test tubes were prepared, each containing 2.9 ml of ONR7a medium. To one set of tubes, phenanthrene was added, whereas naphthalene was added to the second set. All six test tubes were then inoculated with 100 µl of the test strain. Uninoculated controls, acid-killed controls and tubes that were inoculated, but without any added PAH, were also prepared. All test tubes were incubated in the dark with gentle shaking (100 r.p.m.) at 21 °C. PAH degradation was determined spectrophotometrically. For this, the test tubes from each PAH incubation were sacrificed after 14 days for extraction with ethyl acetate (high-pressure liquid chromatography (HPLC) grade). This was performed by adding 2 ml of ethyl acetate to each tube and then vortexing for 30s. Aliquots of the non-aqueous top layer were diluted with ethyl acetate in quartz cuvettes for spectrophotometric analysis at 251 nm for phenanthrene and 275 nm for naphthalene. The A₂₅₁ and A₂₇₅ values were converted to concentrations of phenanthrene and naphthalene, respectively, using the molar absorptivity coefficients of 6.3 x 10⁴ and 5.7 x 10³ mol⁻¹cm⁻¹ (Thomas and Burgess, 2007). A significant decrease (P<0.05) in the PAH concentration measured in the inoculated test tubes, relative to the uninoculated controls, was indicative of degradation.

The ability of the strains to grow on n-hexadecane as the sole carbon and energy source was evaluated in 250-ml Erlenmeyer flasks containing 50 ml of ONR7a medium and supplemented with the hydrocarbon to 0.5% (v/v). Inocula used were cells grown in ONR7a amended with Na-pyruvate (0.1% w/v) and washed three times prior to use. Growth was measured spectrophotometrically by taking measurements of the culture medium periodically at

an optical density of 600 nm. An increase in optical density of at least five-fold was indicative of growth on n-hexadecane.

Sediment enrichment experiment. Microbial enrichment and cultivation experiments were performed on both oil contaminated and non-contaminated surface sediment samples from different cruises, including 3 contaminated samples characterized by the brown oil-derived fallout layer that settled on the seafloor around the Macondo wellhead in fall 2010 (GIP16, Oct. 2010; MUC19, Nov. 2010; E01801, July 2011), and 4 unpolluted samples that lacked this layer (PE19, May 2010; GIP20, Oct. 2010; MUC20, Nov. 2010; E01402, July 2011) (Table 10). 100 μ l sediment was added into 900 μ l 1x PBS (phosphate buffered saline) solution to make stock sediment slurry for series dilution. Then 200 μ l of the series diluted sediment slurry was added into glass test tubes, in which 3 ml ONR7a medium and 15 μ l 100x marine supplement solution was added in. The surface oil collected from May 2010 R/V Pelican cruise was filtered through 0.22 μ m filters; 100 μ l of this filter sterilized oil was added into each test tube. The tubes were incubated in the dark with shaking (100 r.p.m.) at 4 $^{\circ}$ C and 21 $^{\circ}$ C. After three and half weeks (at 4 $^{\circ}$ C) and one week (at 21 $^{\circ}$ C), the highest dilutions that yielded growth were selected for dilution-to extinction culturing in ONR + crude oil. After repeating this process three times, samples (5 μ l) from the highest dilutions producing growth were streaked onto ONR + crude oil agar plates; 200 μ l of the highest dilutions were also subsampled for DNA extraction. All the highest dilution samples enriched at 21 $^{\circ}$ C, as well as two samples enriched at 4 $^{\circ}$ C (MUC19_D1_2 and MUC19_D1_6) were cloned and sequenced. The enrichment MUC19_D1_2 consisted of 100x diluted MUC19 surface sediment supplied with the filtered crude oil and was incubated at 4 $^{\circ}$ C; the enrichment MUC19_D1_6 consisted of the 10^6 dilution of the MUC19 surface sediment, and was incubated together with MUC19_D1_2 at 4 $^{\circ}$ C. Both MUC19_D1_2

and MUC19_D1_6 solution turned turbid after incubation. Subsamples of the highest dilutions were stored frozen at -80 °C in 30% (v/v) glycerol.

Sample	Ship	Date	Depth (m)	Latitude (N)	Longitude (W)	Oil polluted (Y/N)	Sample layer
PE19	RV <i>Pelican</i>	5/7/2010	1605	28°56.962	88°49.777	N	0-3 cm
GIP16	RV <i>Cape Hatteras</i>	10/16/2010	1560	28°43.383	88°24.577	Y	0-1 cm
GIP20	RV <i>Cape Hatteras</i>	10/19/2010	1760	28°45.393	88°9.595	N	0-1 cm
MUC19	RV <i>Atlantis</i>	11/30/2010	1574	28°43.350	88°21.770	Y	0-2.5 cm
MUC20	RV <i>Atlantis</i>	12/1/2010	1885	28°29.290	88°19.050	N	0-2.5 cm
E01801	RV <i>Endeavor</i>	7/25/2011	1630	28°42.382	88°21.815	Y	0-2 cm
E01402	RV <i>Endeavor</i>	7/21/2011	64	28°20.919	91°49.563	N	0-2 cm

Table 10. Sediment samples collected on multiple research cruises near the Macondo wellhead for enrichment cultivation.

4.3 Results

4.3.1 Water column isolation experiment

Hydrocarbon-degrading bacteria were isolated from contaminated surface and plume water samples collected during the Gulf oil spill with and without enrichment on phenanthrene, naphthalene or n-hexadecane. The near-complete 16S rRNA gene sequence (>1400 bp) of each of these isolates was used to construct a phylogenetic tree with related sequences (Figure 30). The tree also presents the potential of the isolates to utilize phenanthrene, naphthalene and/or n-hexadecane as a sole source of carbon and energy for growth. Contaminated surface water samples that were spread on agar plates and sprayed with anthracene or pyrene did not yield colonies that formed clearing zones. Small inconspicuous colonies with faint clearing zones appeared on plates sprayed with fluorine; however, these organisms proved difficult to culture as colonies did not grow out upon further subculturing. Our attempt to isolate a member of the *Oceanospirillales* from the plume, in order to interrogate its nutritional profile for various hydrocarbon substrates, was not successful.

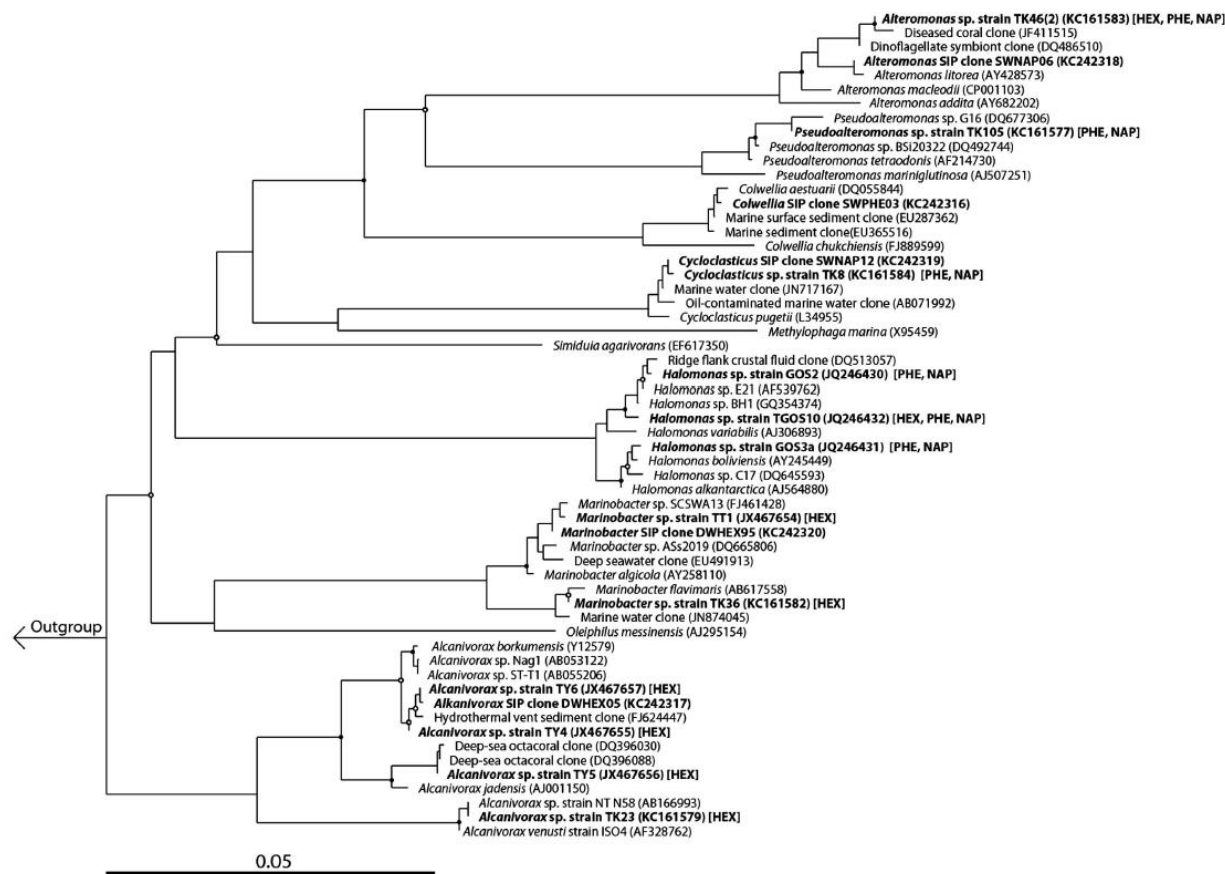


Figure 30. Phylogenetic tree of SIP clones and isolated strains from surface and plume waters. SIP clones and isolates are shown in bold along with the highest similarity sequences and type strains. A neighbor-joining tree was constructed with Jukes–Cantor correction and bootstrap replication (n=1000). Nodes with bootstrap support of at least 65% (○) and 90% (●) are marked. Accession numbers of all sequences and the hydrocarbon(s) utilized (NAP, naphthalene; PHE, phenanthrene; HEX, n-hexadecane) by the isolated strains are given in parentheses. *Akkermansia muciniphila* (CP001071) and *Thalassospira xiamenensis* (AY189753) were used as the outgroup. (Gutierrez *et al.*, 2013)

The 16S rRNA gene sequences of the isolated hydrocarbon-degrading strains were compared with related GenBank sequences and those from related studies (Hazen *et al.*, 2010, Valentine *et al.*, 2010, Baelum *et al.*, 2012, Redmond & Valentine, 2012) (Figure 30). Our cultured *Pseudoalteromonas* strain TK-105 and a *Pseudoalteromonas* sequences (accession number: AY646431) has sequence similarity >98%; the latter represent

Pseudoalteromonadaceae-affiliated sequences found enriched in plume water samples from May 2010 (Hazen *et al.*, 2010). The isolate *Cycloclasticus* strain TK-8 was similar to two *Cycloclasticus* plume clones, H24-8 and H24-11, from plume samples collected in June 2010 (Valentine *et al.*, 2010) (98% and 97% similarity, respectively), as well as to our *Cycloclasticus* clones that dominated in the surface oil slick (98% similarity). The *Marinobacter* strain TT1 and *Halomonas* strains GOS-2, GOS-3a and TGOS-10, however, all shared <97% similarity to respective family-level representative sequences reported in other studies of the Deepwater Horizon blowout (Hazen *et al.*, 2010, Valentine *et al.*, 2010, Redmond & Valentine, 2012). *Alteromonas* strain TK-46(2) represented 0.98% of the sequences in the pyrosequenced surface slick sample (Chapter 1) and shared >97% sequence identity to 60.5% of all *Alteromonas* reads in that library (Table 11). The collective representation of these *Alteromonas* sequences among the total *Alteromonas* pyrosequencing reads in the post-spill water column libraries (C4B4 and C4B8) was >88%; lower but substantial representation was found in the GIP22 control library (40%). The three *Halomonas* strains GOS-2, GOS-3a and TGOS-10 collectively represented 86% of total *Halomonas* reads in the oil slick pyrosequencing library (*Halomonas* sequences were 0.14% of the total library; Table 11), and remained undetectable in the subsurface water column libraries (B1, B3, B6, B11). They accounted for 100%, 63% and 50% of the *Halomonas* pyrosequencing reads in the post-spill samples C4B8, C4B4 and GIP22, respectively. Interestingly, none of the previous studies profiling the microbial response to the oil spill had reported a single *Alcanivorax* sequence in their 16S rRNA pyrosequencing or conventional clone library data. The cultured *Alcanivorax* strains TY4 and TY6 matched (within 97% identity) the single *Alcanivorax* read identified in the surface slick pyrosequencing library. Pyrosequencing reads affiliated to this genus were also identified in the September 2010 (C4B4, C4B8) and

October 2010 (GIP22) post-spill water column samples (Table 11). While the *Alcanivorax* pyrosequencing reads exhibited <97% sequence identity to the cultured strains TY4 and TY6, many (50%, 41% and 25%) of total *Alcanivorax* reads in the C4B8, C4B4 and GIP22 post-spill libraries, showed >97% identity with the sequence of the other cultured *Alcanivorax* sp. isolated in this study, strain TY5.

Isolates	During spill (May 2010)					After spill (Sep/Oct 2010)		
	PE5 (0 m)	B1 (1320 m)	B3 (1210 m)	B6 (1170 m)	B11 (800 m)	C4B8 (800 m)	C4B4 (1210 m)	GIP22 (1050 m)
From surface oily water								
<i>Cycloclasticus</i> TK-8	94.1 (95.42) ^b	2.8 (3.17)	6.6 (6.55)	12.9 (14.38)	0.79 (0.79)	0.21 (0.21)	5.3 (5.7)	0.5 (0.91)
<i>Alteromonas</i> TK-46(2)	0.98 (1.45)	0 (0)	0 (0)	0 (0)	0 (0)	1.31 (1.48)	1.85 (2.18)	0.08 (0.2)
<i>Halomonas</i> GOS-2, GOS-3a, TGOS-10	0.12 (0.14)	0 (0)	0 (0)	0 (0)	0 (0)	0.04 (0.04)	0.05 (0.08)	0.02 (0.04)
<i>Marinobacter</i> TK-36	0 (0.02)	0 (0)	0 (0)	0 (0)	0 (0.79)	0.2 (0.7)	0.01 (0.2)	0.06 (0.1)
From plume water								
<i>Alcanivorax</i> TY4, TY6	0.004 (0.004)	0 (0)	0 (0.24)	0 (0)	0 (0)	0 (0.12)	0 (0.17)	0 (0.28)
<i>Alcanivorax</i> TY5	0	0	0	0	0	0.06	0.07	0.07
<i>Marinobacter</i> TT1	0 (0.02)	0 (0)	0 (0)	0 (0)	0 (0.79)	0.2 (0.7)	0.01 (0.2)	0.06 (0.1)
<i>Pseudoalteromonas</i> TK-105	1.0 (1.2)	0	0	0	0	0 (0.1)	0 (0.11)	0 (0.04)

Table 11. Relative abundance (%) of the isolated strains in 16S rRNA gene pyrosequencing libraries^a.^aPercentage of total reads in each library—refer to pyrosequencing table in Chapter 1. No matches were identified in any of the libraries for *Colwellia* OTU-2 and *Alcanivorax* TK-23.^bValues in parentheses represent the % of sequences affiliated to the respective genus relative to the total number of sequences in that library. (Gutierrez *et al.*, 2013)

4.3.2 21 °C sediment enrichment

The enrichments in the highest dilution samples of all 7 sediments (oil polluted and non-polluted sediments) (Figure 31) were able to degrade oil by emulsifying added crude oil in few days. However, our attempt of isolating the oil-degrading bacteria colonies on ONR + crude oil plates failed as no visible colonies grew on the plates in 2 weeks. Thus, the 7 highest enrichments samples were cloned and sequenced to identify possible oil degraders. As shown in Table 12 and Figure 32, the enriched oil degrading bacteria candidates fall mostly within genera and lineages of the *Gammaproteobacteria* (including *Alcanivorax*, *Thalassolituus* and its sister group, *Pseudomonas*, *Marinobacter*, and an uncultured gammaproteobacteria clade GU584754), while few sequences are members of the *Alphaproteobacteria* (*Hyphomonas*) and the

Bacteroidetes (*Balnola*). Various species of *Alcanivorax* and *Thalassolituus* composed the majority of all the 7 enrichments. The similarity of the enrichments of different sediment samples maybe caused by the same carbon source (crude oil) we added into each sample. However, the *Alcanivorax* clade represented by *Alcanivorax borkumensis* was only obtained in the E01402 enrichment, and the clade 99% similar to *Alcanivorax venustensis* was found only in the E01801 enrichment. Similarly, the *Marinobacter* group was only found in the PE19 enrichment, while *Pseudomonas* group was obtained from the E01402 enrichment. These divergent enrichment results indicate differences between the original sediment bacterial communities.

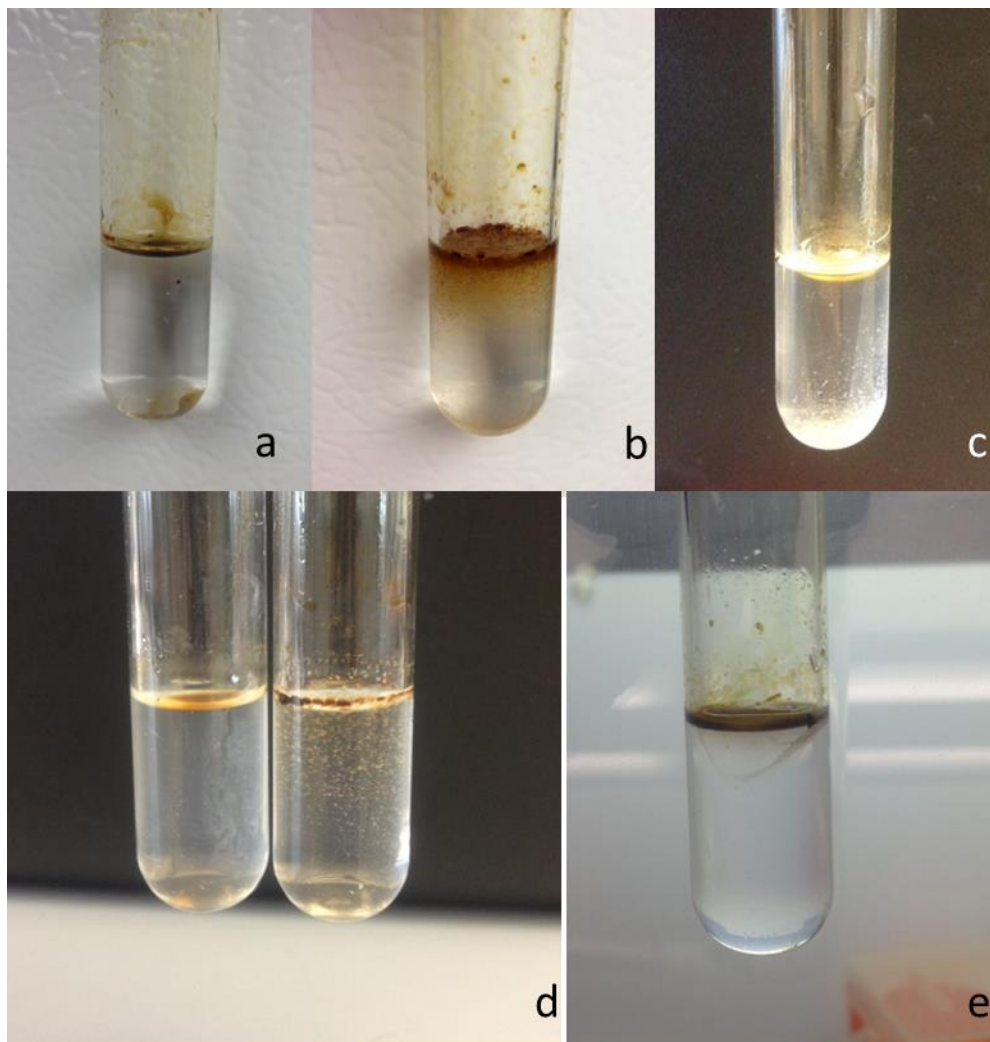


Figure 31 enrichment of oil degraders from sediment samples under 21 °C. a: freshly diluted sediment amended with filtered crude oil at day 0. b-e: oil-degradation started and the enrichments turned turbid after incubated for 2-5 days at 21 °C. In picture e, a white floc formed in 5 days. Notice the oil emulsification and color of the enrichments between samples are different.

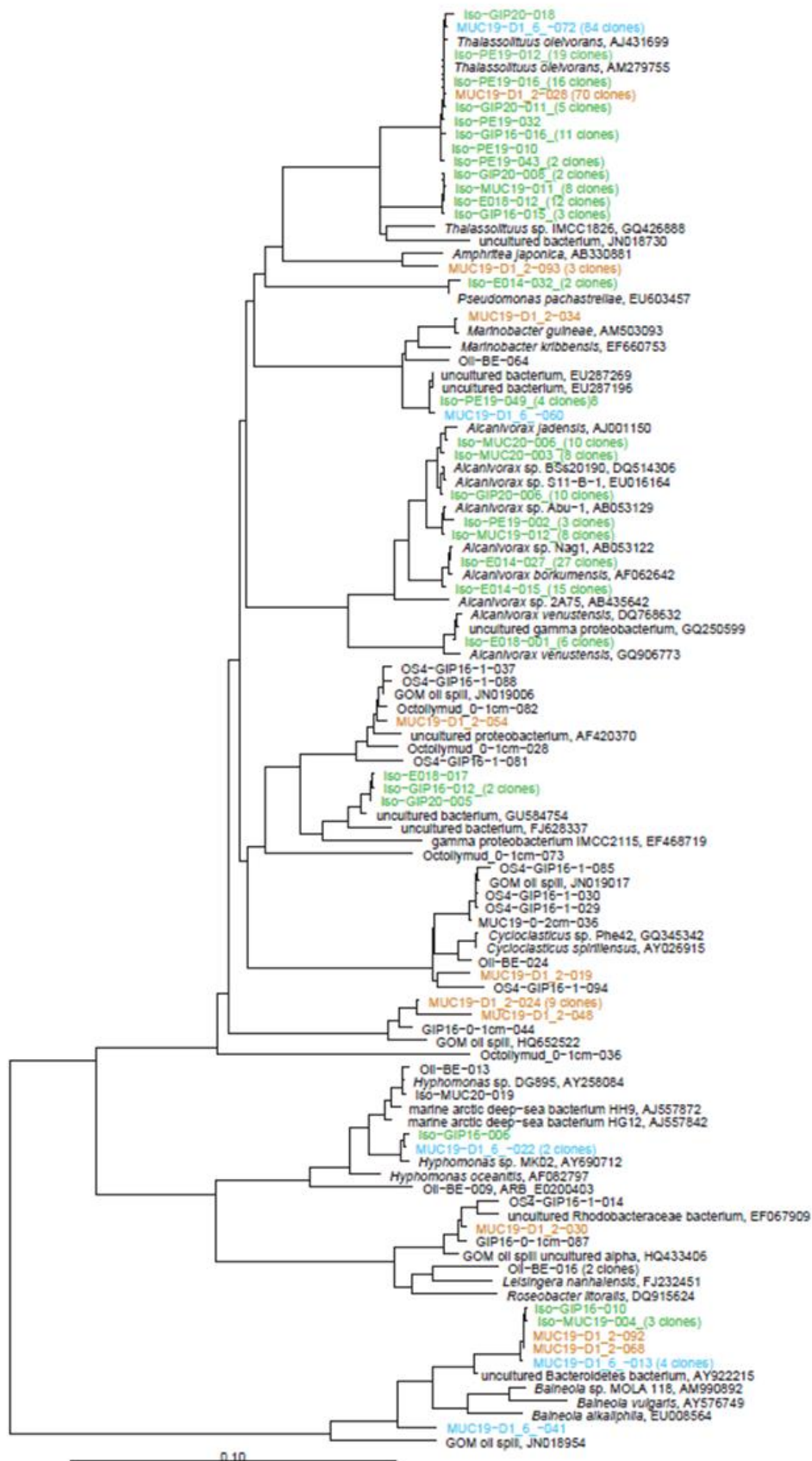


Figure 32. Phylogenetic tree of clones from 4 °C and 21 °C sediment enrichments. The 4 °C enriched sample MUC19_D1_2 was labeled in orange color, sample MUC19_D1_6 was labeled in blue; all the 21 °C enrichment clones from 7 sediments were labeled in green.

Sample name	PE19	GIP16	GIP20	MUC19	MUC20	E018	E014
Total clone numbers	46 clones	18 clones	19 clones	19 clones	19 clones	19 clones	44 clones
Alcanivorax sp. Abu-1	3 (99%)			8 (99%)			
Alcanivorax jadensis			10 (100%)		18 (99%)		
Alcanivorax borkumensis							15 (99%)
Alcanivorax sp. Nag1							27 (99%)
Alcanivorax venustensis						6 (99%)	
uncultured Marinobacter (EU287269)	4 (100%)						
Thalassolituus oleivorans	39 (100%)	11 (99%)	6 (99%)				
Thalassolituus oleivorans (sister group)		3 (96%)	2 (96%)	8 (96%)		12 (96%)	
Hyphomonas		1 (99%)			1 (99%)		
Balneola		1 (96%)		3 (96%)			
Pseudomonas							2 (99%)
Uncultured Gamma* (GU584754)		2 (99%)	1 (99%)			1 (99%)	

Table 12. Clone libraries of room temperature (21 °C) sediment enrichments. Sequence similarity to the reference sequence is shown in parenthesis. * The clone GU584754 affiliated with previous published GOM oil spill clones (Redmond & Valentine, 2012)

4.3.3 4 °C sediment enrichment

Bacterial growth at low temperature was much slower than at room temperature. After continuing the enrichment experiment for 85 days, we checked the cold-enriched samples from MUC19, MUC19-D1-2 and MUC19-D1-6, with clone library analyses. MUC19-D1-2 was prepared by mixing the 100 times diluted original sediment with 3 ml ONR media and 100 µl crude oil; MUC19-D1-6 was 10⁴ diluted from MUC19-D1-2, and represents the highest dilution of serially diluted MUC19 sediment that retained oil degradation ability at 4 °C. In these two cold enrichments, 70 out of 90 clones in MUC19-D1-2 and 84 out of 92 clones in MUC19-D1-6 are highly similar (99% similarity) to *Thalassolituus oleivorans*; 2 clones of MUC19-D1-2 and 5 clones of MUC19-D1-6 were found affiliated to the *Balneola* group (95%-96% similarity) (Figure 32). The remaining 3 clones of MUC19-D1-6 were within a *Marinobacter guineae* sister

group (1 clone) and in the *Hyphomonas* sp. MK02 group (2 clones, 99% similarity to the representative sequence EU287269), closely related to the PE19 and GIP16 room temperature enrichment, respectively (Figure 32). Compare to the highly diluted MUC19-D1-6, the less diluted cold enrichment MUC19-D1-2 contained more diversity in its clone library. Beside the *Thalassolituus* and *Balneola* clone, the remaining 20 clones of MUC19-D1-2 were members of the genera *Marinobacter guineae* (1 clone, 99% similarity), *Cycloclasticus* (1 clone, 97% similarity to the dominant *Cycloclasticus* sequence JX878969 derived from surface oil slick, 98% similarity to *Cycloclasticus spirillensus*), *Amphritea* (3 clones, 98% similarity), of two uncultured gammaproteobacterial groups represented by the hydrocarbon plume sequence HQ652522 (Valentine *et al.*, 2010) (9 clones with 98% similarity, 1 clone with 96% similarity) and the sequence JN019006 from the spilled oil contaminated water column (Redmond & Valentine, 2012) (1 clone, 99% similarity), and of an uncultured Alphaproteobacterial marine *Roseobacter* cluster (1 clone, 99% similarity to sequence HQ433406, which was also retrieved from the plume). With the exception of the *Amphritea* group, the MUC19-D1-2 clones were affiliated with sequences derived from the oil spill contaminated water column, polluted surface sediment or the oily marine snow particles.

4.4 Discussion

4.4.1 Water sample enrichment

Using cultivation-based methods, coupled with DNA-SIP (Gutierrez *et al.*, 2013), we identified several phylotypes affiliated to hydrocarbon-degrading bacterial genera, namely *Cycloclasticus*, *Alteromonas* and *Halomonas*, from surface oil slick and plume waters (Figure 33). Cross-checking the 16S rRNA sequences of these isolates and SIP-identified clones against water column pyrosequencing data revealed that these were the members of the same lineages with the dominant hydrocarbon-degraders in contaminated surface waters and, to a lesser extent,

in the plume during the active phase of the spill. Drawing our results together: the ability of *Cycloclasticus* strain TK-8 to degrade hydrocarbons, the observed dominance of highly similar *Cycloclasticus* sequences in clone libraries constructed from ^{13}C -enriched DNA (^{13}C labeled phenanthrene and naphthalene enrichments), and the dominance of this genus in the surface oil slick clone library and pyrosequencing library, it is clear that *Cycloclasticus* (strain TK-8 as cultured representative) had a major role in the degradation of aromatic hydrocarbons in surface water. This concurs with the fact that members belonging to this genus are recognized for their distinct specialization in degrading aromatic hydrocarbons almost exclusively as a sole source of carbon and energy (Head *et al.*, 2006, Yakimov *et al.*, 2007). Enrichment of these sequences in the plume pyrosequencing libraries indicates that *Cycloclasticus* may have also contributed significantly to the degradation of aromatic hydrocarbons in deeper waters (Figure 33).

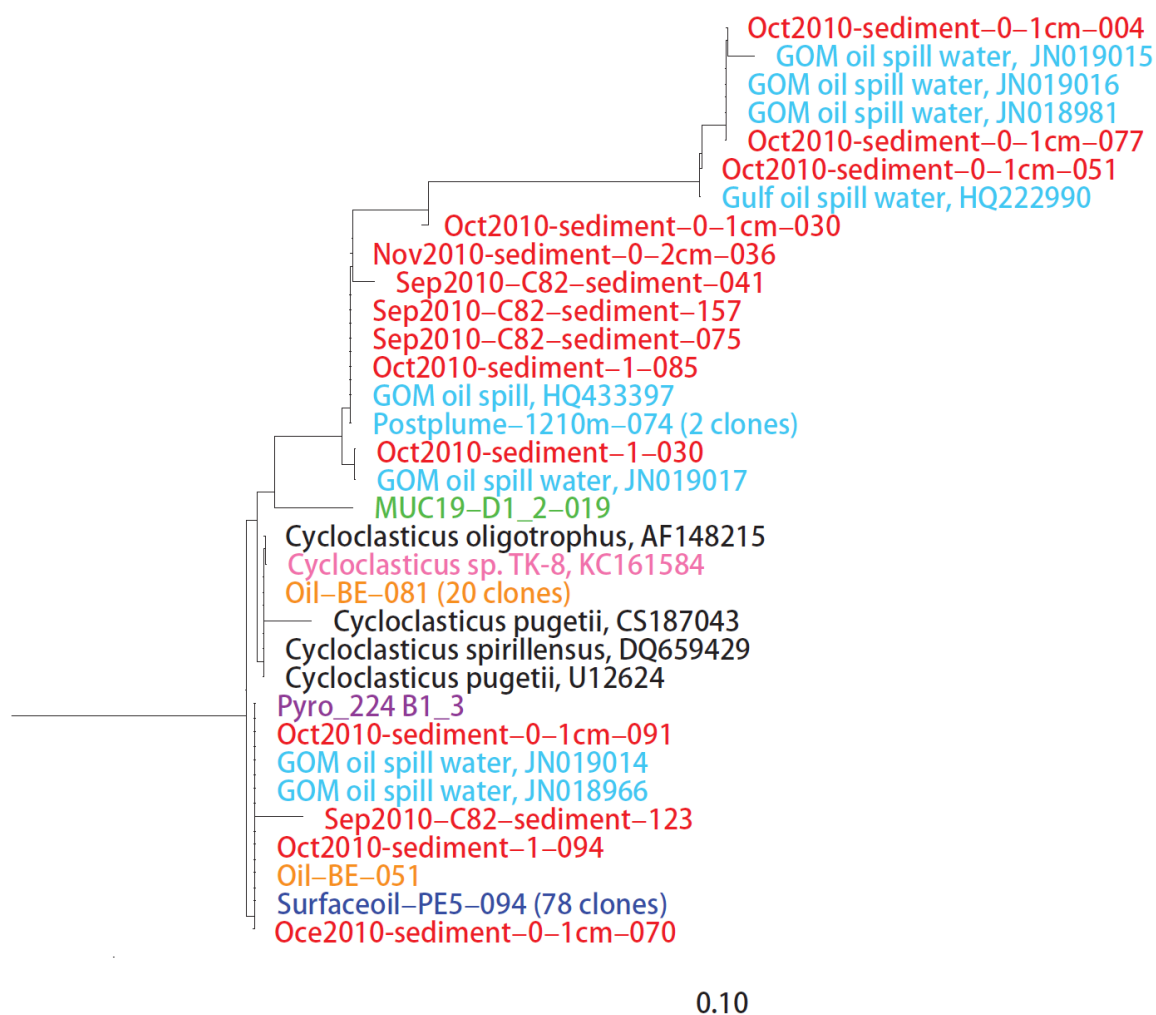


Figure 33. Phylogenetic tree of genus *Cycloclasticus*. Red: sequences from oil contaminated sediment from different time points. Bright blue: sequences from post-plume water column. Green: sequence from 4 °C enrichment. Pink: pure cultured *Cycloclasticus* strain from surface oil slick. Purple: sequence fragment from pyrosequencing library. Orange: roller bottle oil aggregates' sequence. Dark blue: sequences from surface oil slick.

To our knowledge, no report has hitherto emerged identifying *Alcanivorax* in the water column during the spill. This is interesting from the point of view that members of this group are often strongly selected for in oil-impacted environments (Head *et al.*, 2006, Yakimov *et al.*, 2007). Since *Alcanivorax* spp. preferentially degrade branched- and/or straight-chain saturated

hydrocarbons (Head *et al.*, 2003) that constitute a large fraction of light crude oils, such as the one that entered into the Gulf of Mexico from the leaky Macondo MC252 well (Reddy *et al.*, 2012), members of genus could be expected to have bloomed during the DWH spill. Using SIP and cultivation-based methods, we identified a number of *Alcanivorax* phylotypes in both surface slick and plume water samples, hence, revealing that members of this genus were present in the water column during the spill (Figure 34). Cross-checking their abundance against the water column pyrosequencing data showed that members of this genus were present at very low levels in the surface slick (PE5) and plume water samples (B3), and similarly in all the post-spill samples (Table 11). It is possible the growth of *Alcanivorax* was restrained by the low temperature and high pressure in the deep plume, and was outcompeted by *Cycloclasticus* in the surface slick as well as the uncultured *Oceanospirillales* in the plume.

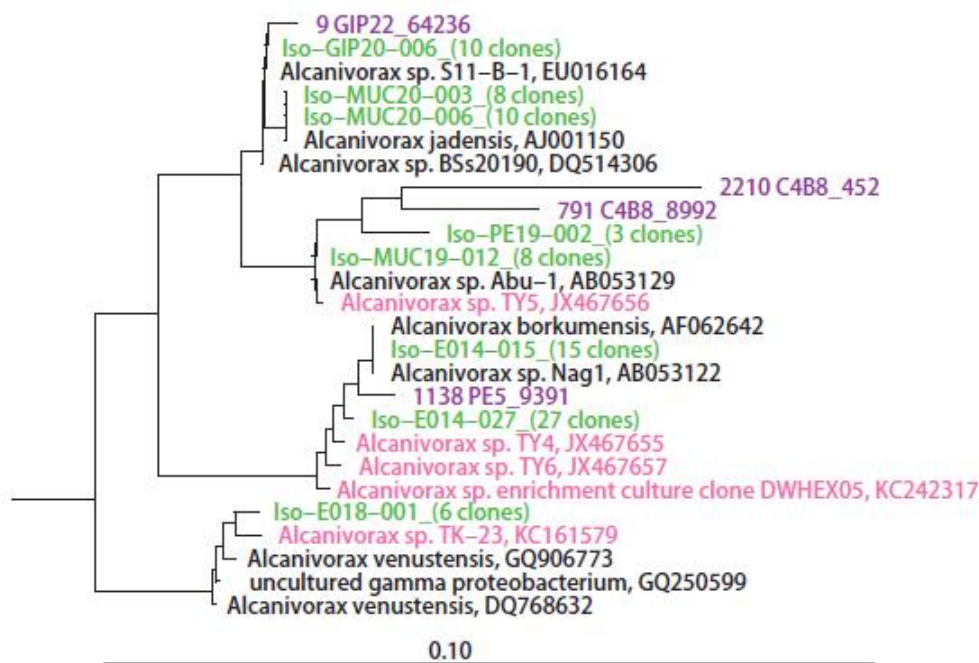


Figure 34. Phylogenetic tree of genus *Alcanivorax*. Green: sequence from 4 °C and 21 °C enrichments. Pink: pure cultured *Alcanivorax* strains and SIP enrichment sequence from the deep plume. Purple: sequence fragment from pyrosequencing libraries.

4.4.2 Sediment samples enrichment

Thalassolituus. The majority of enriched sediment clones was affiliated to the two main genera *Thalassolituus* and *Alcanivorax*. *Thalassolituus* and *Alcanivorax* are obligate oil-degrading marine bacteria (OHCB, characterized by their ability to metabolize only a very restricted spectrum of carbon substrates) within the *Gammaproteobacteria* (Yakimov *et al.*, 2007). The type strain of *Thalassolituus*, *Thalassolituus oleivorans*, was isolated at 20 °C from tetradecane-amended shallow water seawater/sediment samples collected from the harbor of Milazzo, Sicily, Italy. The obligately aerobic, motile species *T. oleivorans* grows almost exclusively on aliphatic hydrocarbons and their derivative, fatty acids and fatty alcohols (Yakimov *et al.*, 2004, Golyshin *et al.*, 2013). *Thalassolituus* has been confirmed to play an important role in crude oil degradation, and has been found in both cold marine environments, such as Arctic Ocean and Antarctic coastal area, as well as in temperate locations of the Black and Mediterranean Seas (Golyshin *et al.*, 2013). The fact that *T. oleivorans* lives in both warm and cold habitats is coincident with our results: enrichments from both temperatures closely clustered with strains of *T. oleivorans* (99% similarity); the clones from 4 °C enrichments are identical to the 21 °C clones. In our 21 °C enrichments, some clones formed a sister group of *T. oleivorans* (96% similarity) even though the substrate was the same. If pure cultures of this cluster existed, they could be used to examine the question whether the two groups of *Thalassolituus* prefer different components of the crude oil.

Alcanivorax. The *Alcanivorax* clade was represented only by clones from 21 °C enrichments; except GIP16, all of the remaining 6 tested sediments obtained *Alcanivorax* clones, related to the species *Alcanivorax jadensis*, *Alcanivorax venustensis* (only clones from E01801) and *Alcanivorax borkumensis* (only clones from E01402) (Figure 34). *A. jadensis* was originally

isolated from the intertidal sediment of the North Sea coast at 25 °C, supplemented with hexadecane as the sole carbon source (Bruns & Berthe-Corti, 1999). *A. jadensis* is able to grow aerobically as well as anaerobically by nitrate reduction. It grows with pyruvate, acetate, as well as long chain alkanes like tetradecane, hexadecane and pristane (Bruns & Berthe-Corti, 1999). *A. venustensis* was isolated from filtered Mediterranean seawater; it utilizes tetradecane, hexadecane, as well as organic acids such as pyruvate, propionate, acetate and hydroxybutyrate (Fernandez-Martinez *et al.*, 2003). *A. borkumensis*, the model organism of OHCB, was derived from the Western-Elms harbor in the North Sea; it is capable to grow on many saturated petroleum fraction constituents and on biogenic hydrocarbons: straight-chain (up to C32 in length) and branched alkanes, isoprenoids and long side-chain alkyl-aromatic compounds (Dutta & Harayama, 2001, Yakimov *et al.*, 2007). This maybe the presumed reason why *A. borkumensis* has been found in all types of marine environments as well as a few terrestrial environments that share salinity and the presence of hydrocarbons with the marine ecosystem (Yakimov *et al.*, 2007). Genomic study of the *A. borkumensis* type strain SK2 has revealed the co-existence of three different alkane-oxidizing systems, namely two alkane hydroxylase systems AlkB1 and AlkB2 and three P450 cytochromes; all of the systems involved in catabolism of saturated hydrocarbons as suggested by proteomic profiling (Hara *et al.*, 2004, van Beilen *et al.*, 2004, Kubota *et al.*, 2005, Sabirova *et al.*, 2006, Schneiker *et al.*, 2006). Moreover, the SK2 genome specifies a large amount of genetic determinants for the uptake of mineral nutrients that are limited in marine environment. Since a sudden input of oil (for example, oil spill) would lead to nutrient imbalances (C/P or, the trace elements required by microbes), genetically *A. borkumensis* is well prepared by obtaining genes active nitrate uptake and reduction and high-affinity transporter systems for other important oligo-nutrients, like phosphate, magnesium and

zinc (Schneiker *et al.*, 2006, Yakimov *et al.*, 2007). All the genetic determinants for scavenging functions enables *A. borkumensis* SK2 to efficiently exploit the attenuate hydrocarbons in marine environment or the adapt to a sudden appearance of hydrocarbons and carbon, such as nutrient imbalances that occur during and after oil spill (Yakimov *et al.*, 2007). Since the substrate for all the enrichment sediments was the same, the result that different sediment samples yielded phylotypes related to at least derived various *Alcanivorax* species may be attributed to the inherent geographical and physical differences of each sample, as well as the differences among the microbial seed populations in the sediment. Notably, in another isolation experiment using the DWH oil contaminated beach sands yield pure cultured *Alcanivorax* strains with 100% identical 16S rRNA sequence to the different species *A. dieselolei*, which was not found in our enrichments (Kostka *et al.*, 2011).

Marinobacter and others. The genus *Marinobacter* contains several alkane degrading species. A MUC19-D1-2 enrichment clone was closely related to the species *M. guineae*, which has not been tested for hydrocarbon degradation, but is able to hydrolyze the Tween (polyoxyethylene sorbate) compound Tween 80 that is considered diagnostic of the substrate specificity of marine bacteria toward the use of hydrocarbons as a source of carbon and energy (Yakimov *et al.*, 2007). The *Marinobacter* clones found in the PE19 and MUC19-D1-6 enrichment also belong to a sister group of *M. guineae* originally from Antarctica marine sediment (Montes *et al.*, 2008), and differ from our plume water isolate and the *Marinobacter* strains isolated from oiled beach sands (Kostka *et al.*, 2011) as well as our plume and SIP isolates (Gutierrez *et al.*, 2013) (Figure 35). The different oil sources as well as the intrinsic heterogeneities of sediment and sand may stimulate different indigenous microbial seed populations. However, the sediment enrichment and the sand study also share some minority

groups. For example, a *Pseudomonas pachastrellae* strain isolated from a marine sponge was able to degrade Tween 80 (Romanenko *et al.*, 2005).

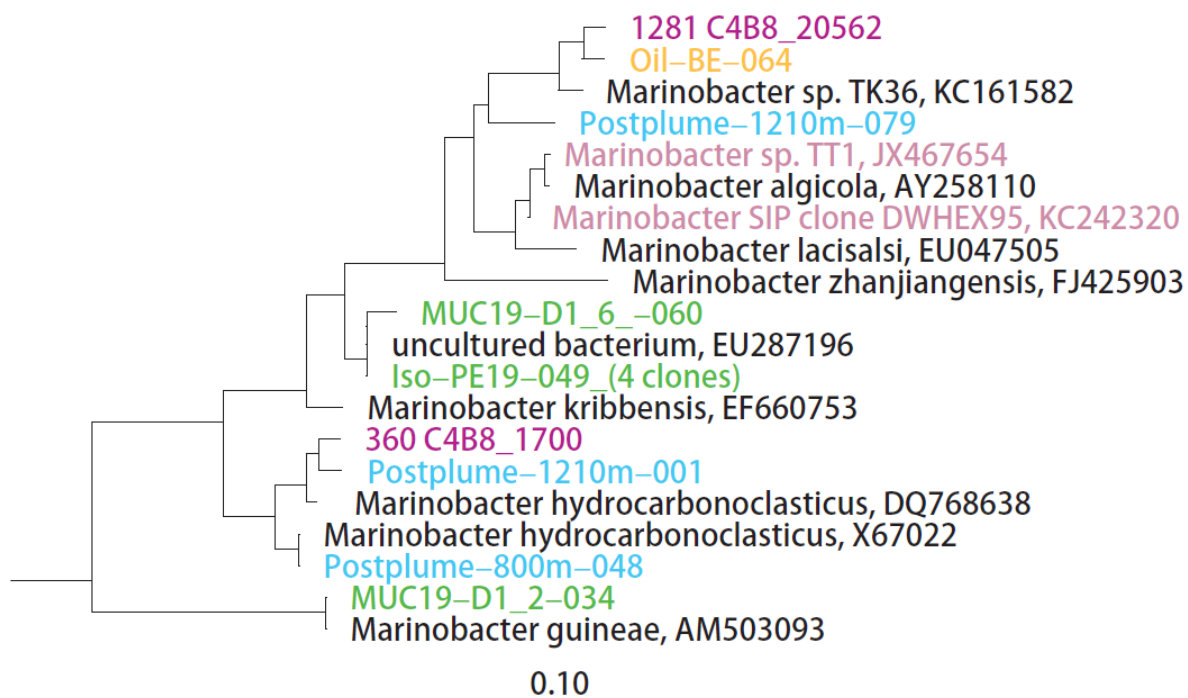


Figure 35. Phylogenetic tree of genus *Marinobacter*. Bright blue: sequences from post-plume water column. Green: sequence from 4 °C and 21 °C enrichments. Pink: pure cultured *Marinobacter* strain and SIP enrichment from the deep plume. Purple: sequence fragment from pyrosequencing libraries. Orange: roller bottle oil aggregates' sequence.

The enriched *Gammaproteobacteria* clones (from GIP16, GIP20 and E018) contains an uncultured group which is of special interests, since its most closely related to uncultured bacteria group potentially oxidizing propane (main component of the deep hydrocarbon plume during spill) as detected by SIP experiment (Redmond *et al.*, 2010). Besides *Gammaproteobacteria*, few enrichment clones affiliated to genus *Hyphomonas* of *Alphaproteobacteria* and genus *Balneola* of *Bacteroidetes*. The uncultured *Hyphomonas* clone most closely related to our enrichment was from heavily oil polluted soil (Liu *et al.*, 2009), while

the uncultured *Balneola* clone was derived from a whale fall affected sediment (Tringe *et al.*, 2005) which contained high content of organic matter.

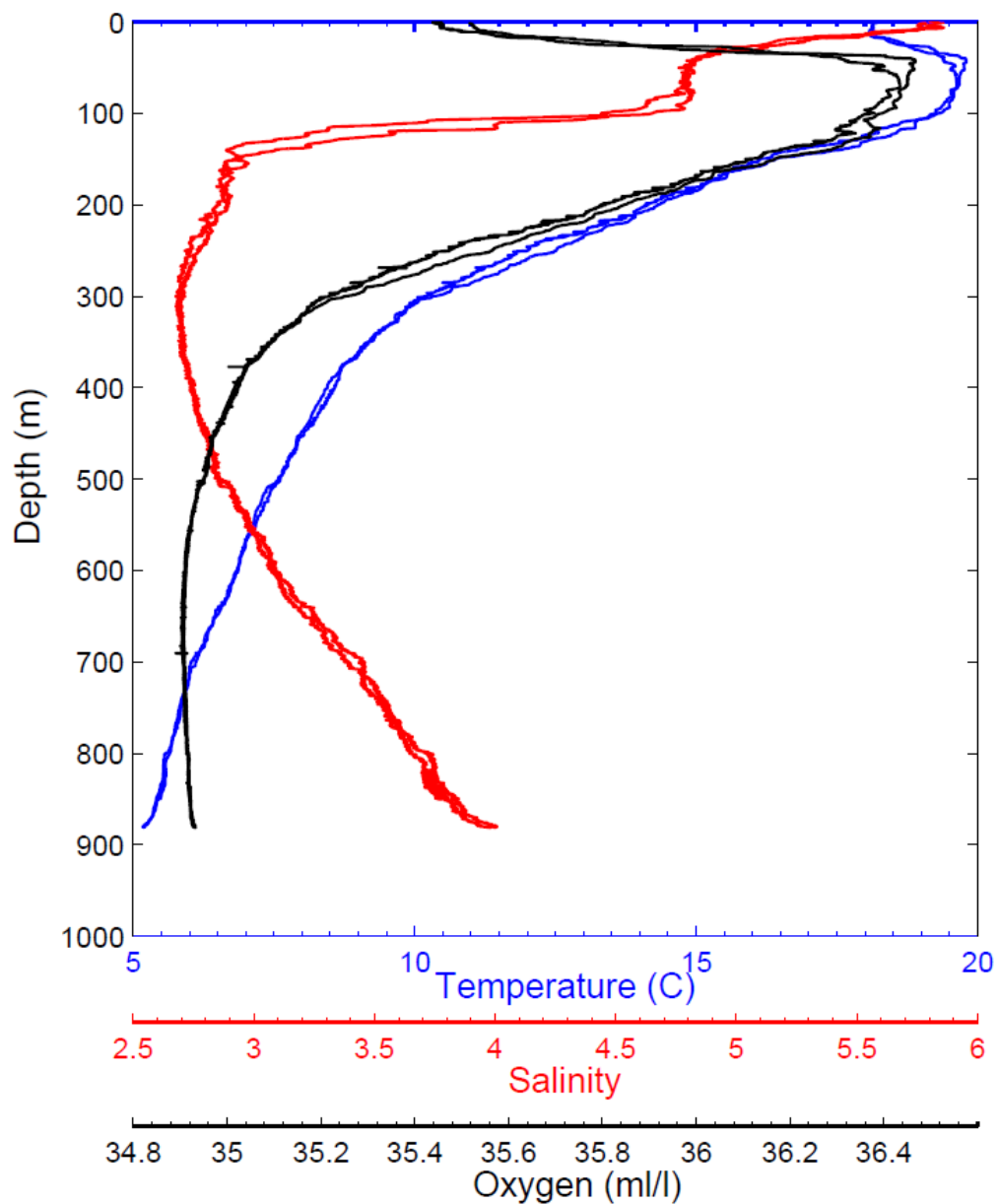
Beside the sequences that highly similar to cultured species, those MUC19-D1-2 enrichment clones that affiliated to uncultured groups are also informative. The 2 uncultured gammaproteobacterial groups contain MUC19-D1-2 sequences are not only represented by clones derived from hydrocarbon plume but also include many sequences from the October and/or September 2010 oily surface sediment clone library (>99% similar to the MUC19-D1-2 sequences) (Fig 4.3). Moreover, the alphaproteobacterial sequence from the MUC19-D1-2 enrichment fell into *Roseobacter* clade, together with the sequences from September, October 2010 oily surficial sediments (>99% similarity) and those detected within the lab incubated oil marine snow in Chapter 2. Even though the MUC19 sediment 16S rRNA clone library had not indicated dominant oil degraders or sharing potential oil-degrading groups with other oil-polluted samples (Chapter 3), the appearance of the hydrocarbon degraders in MUC19 4 °C enrichment showed the seed populations exist in this sample. These similar uncultured groups between the heavily contaminated samples and MUC19 enrichment further indicated that these groups maybe induced by fresh oil, and can be explained as the fast responder of microbes within MUC19 sediment to the oil input. This response is coincident with our hypothesized bacterial community succession throughout time with the large input, thus the 4 °C enrichment result could be an evidence of our hypothesis in Chapter 3.

4.5 Conclusion

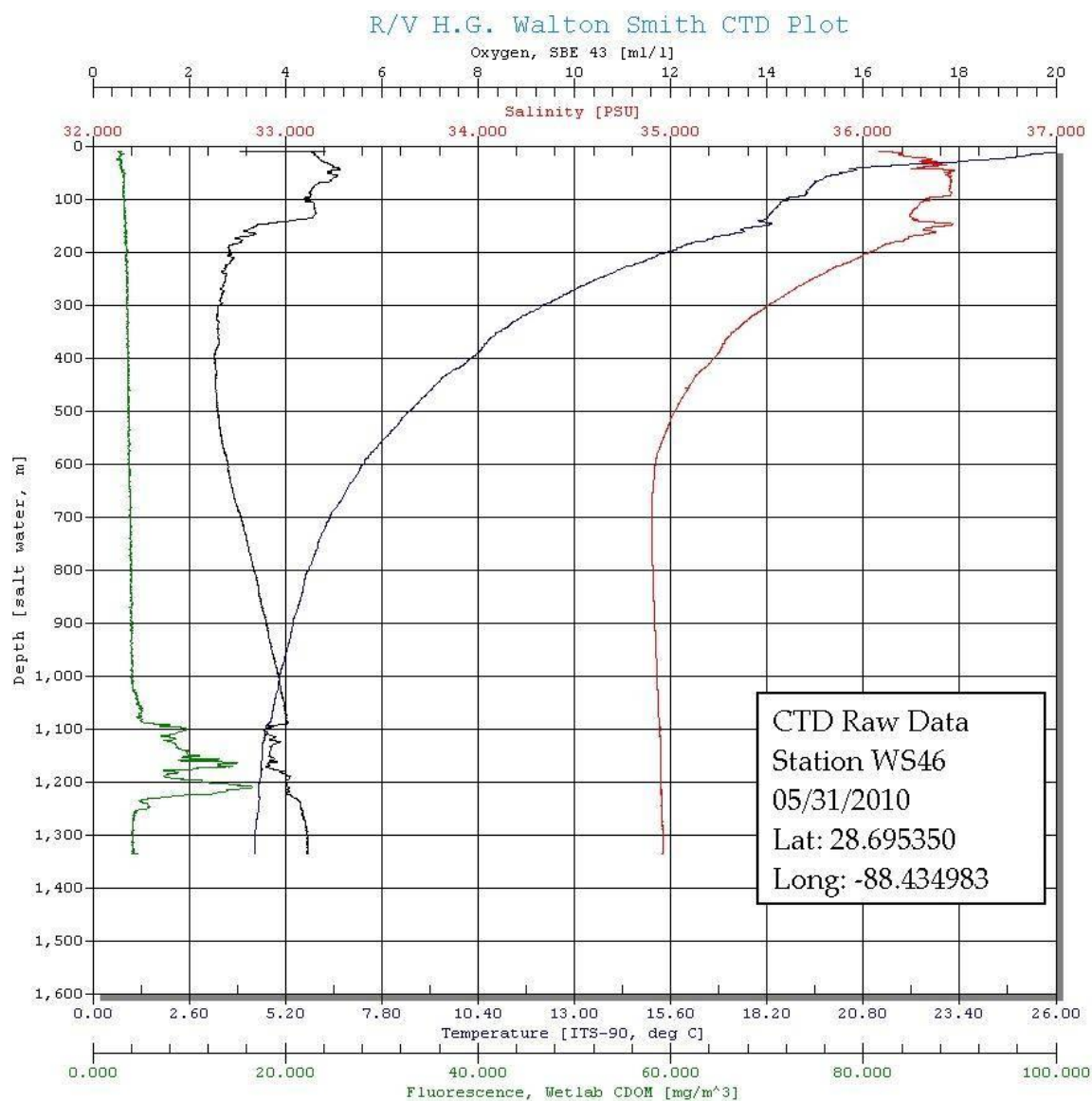
We isolated various hydrocarbon-degrading bacterial strains from the surface oily water (*Cycloclasticus*, *Alteromonas*, *Halomonas* and *Marinobacter*) and the deep hydrocarbon plume (*Alcanivorax*, *Marinobacter* and *Pseudoalteromonas*). Their appearance is corresponding to the environment: in the surface oil slick, PAH was the dominant hydrocarbon source that provide

food to the obligate PAH-degrading bacteria *Cycloclasticus*, while *Alteromonas* and *Halomonas* may also contribute to hydrocarbon degradation, but more importantly, they can produce EPS to dissolve the PAH hydrocarbons and form large oil aggregates, as observed during the spill; while in the deep plume, light hydrocarbons were the dominant carbon source which fit the food spectrum of the obligate alkane degrading bacteria *Alcanivorax* and *Marinobacter*, together with some *Pseudoalteromonas* that can utilize light hydrocarbons. Our dilution-to-extinction sediment enrichments yielded two alkane degrading bacteria as main groups: *Alcanivorax* (21 °C only) and *Thalassolituus* (at 4 °C and 21 °C). Notably, at 4 °C, in the 100 time diluted MUC19 sediment amended with the filtered surface oil, yielded clones similar to those previously found in plume and post-plume water and oil-contaminated October 2010 sediment. These bacteria could be from the degraded oil or from oil-induced sediment, both of which indicate these sequences related to the oil input.

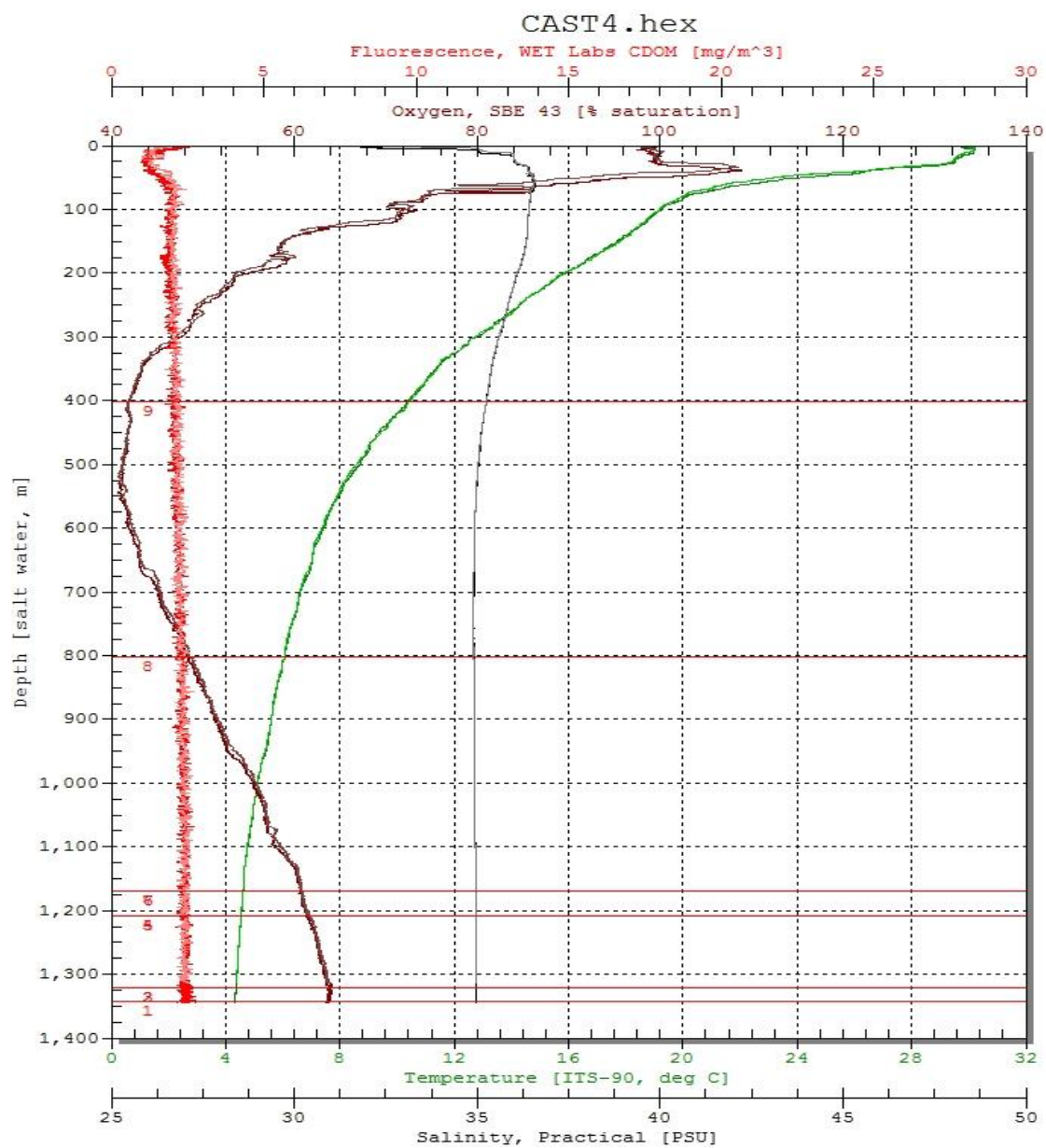
APPENEX



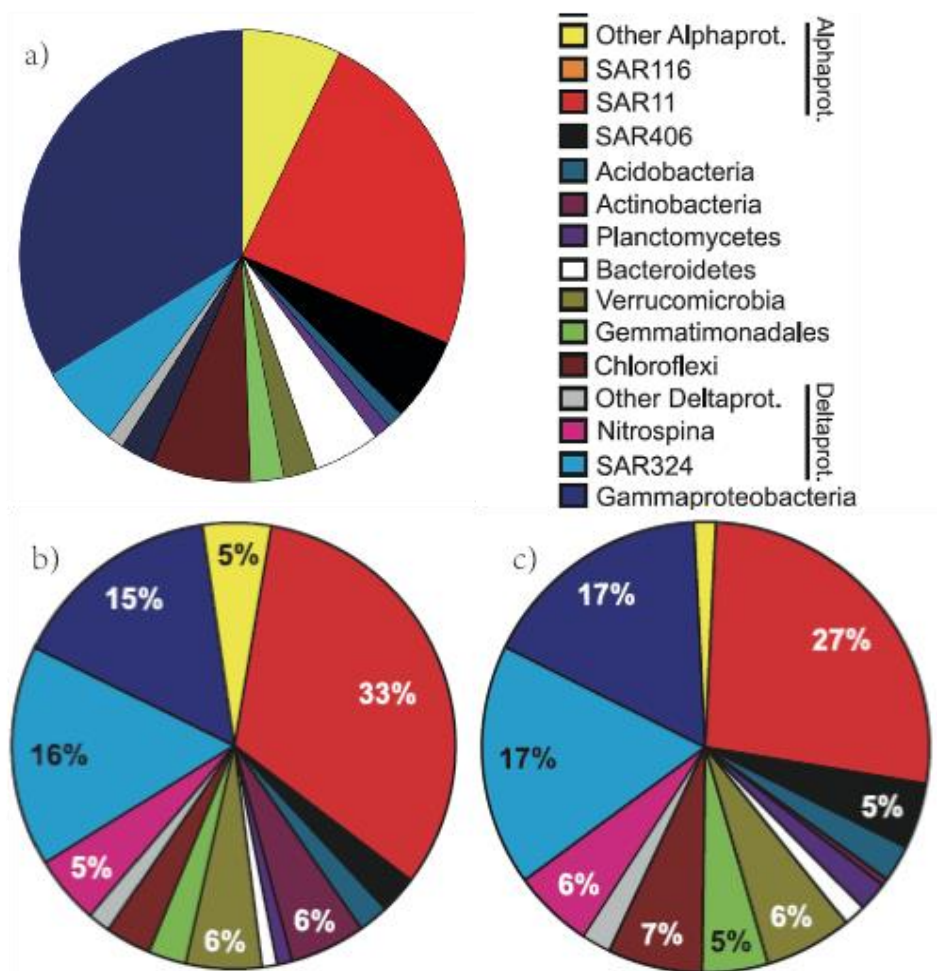
CTD profile of Pre-Plume water column near MC118, March 10, 2010



CTD profile of plume water column near Macondo wellhead, May 31, 2010. Water samples for sequencing analysis were obtained from depths of 800 m (above plume), 1170 m (plume), 1210 m (plume), and 1320 m (below plume).



CTD profile of Post-Plume water column near Macondo wellhead, Sept 12, 2010. Water samples for sequencing analysis were obtained from depths of 800 m and 1210 m.



Comparison of pre-spill 16S rRNA gene clone library near the DWH site, and single cell representation of bacterial communities in the Atlantic and Pacific Ocean based on Swan et al. 2011.

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